CRYSTALLIZATION OF IGF-1

Background of the Invention

Related Applications

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60\267,977 filed February 9, 2001, and provisional application no. 60/287,072 filed April 27, 2001, the contents of which are incorporated herein by reference.

Field of the Invention

This invention is directed to a crystalline form of human insulin-like growth factor-1 (IGF-1) and more particularly to a crystal of human IGF-1, a method of crystallization thereof, and its structure, obtained by x-ray diffraction. In addition, the invention relates to methods of identifying new IGF-1 agonist molecules based on biophysical and biochemical data suggesting that a single detergent molecule that contacts residues known to be important for IGF-1 binding protein (IGFBP) interactions binds to IGF-1 specifically, and blocks binding of IGFBP-1 and IGFBP-3.

Description of Related Disclosures

There is a large body of literature on the actions and activities of IGFs (IGF-1, IGF-2, and IGF variants). Human IGF-1 is a serum protein of 70 amino acids and 7649 daltons with a pI of 8.4 (Rinderknecht and Humbel, Proc. Natl. Acad. Sci. USA, 73: 2365 (1976); Rinderknecht and Humbel, J. Biol. Chem., 253: 2769 (1978)) belonging to a family of somatomedins with insulin-like and mitogenic biological activities that modulate the action of growth hormone (GH) (Van Wyk *et al.*, Recent Prog. Horm. Res., 30: 259 (1974); Binoux, Ann. Endocrinol., 41: 157 (1980); Clemmons and Van Wyk, Handbook Exp. Pharmacol., 57: 161 (1981); Baxter, Adv. Clin. Chem., 25: 49 (1986); U.S. Pat. No. 4,988,675; WO 91/03253; WO 93/23071). IGFs share a high sequence identity with insulin, being about 49% identical thereto. Unlike insulin, however, which is synthesized as a precursor protein containing a 33-amino-acid segment known as the C-peptide (which is excised to yield a covalently linked dimer of the remaining A and B chains), IGFs are single polypeptides (see Figure 1).

In the developing embryo, the absence of IGF-1 leads to severe growth retardation that continues post-natally (Baker et al., Cell, 75: 73-82 (1993); Powell-Braxton *et al.*, Genes Dev., 7: 2609-2617 (1993); Liu *et al.*, Cell, 75: 59-72 (1993); Liu *et al.*, Molecular Endocrinol., 12: 1452-1462 (1998)). While most (greater than 75%) of serum IGF-1 is produced by the liver in response to growth hormone, this liver-derived IGF-1 has been shown to be unnecessary for post-natal body growth in mice (Sjogren *et al.*, Proc. Natl. Acad. Sci. USA, 96: 7088-7092 (1999)). Rather, it is the locally produced, non-hepatic IGF-1, acting in a paracrine/autocrine manner, which appears to be responsible for most of the post-natal growth-promoting effects of IGF-1 (Schlechter *et al.*, Proc. Natl. Acad. Sci. USA, 83: 7932-7934 (1986); Isaksson *et al.*, Science, 216: 1237-1239 (1982)). Consistent with its growth-promoting effects, IGF-1 is a powerful mitogen, regulating diverse cellular functions such as cell-cycle progression, apoptosis, and cellular differentiation (reviewed in Jones and Clemmons, Endocr. Rev., 16: 3-34 (1995) and in LeRoith, Endocrinology, 141: 1287-1288 (2000)).

IGFs have been implicated in a variety of cellular functions and disease processes, including cell cycle progression, proliferation, differentiation, and insulin-like effects in insulin-resistant diabetes. Thus,

IGF has been suggested as a therapeutic tool in a variety of diseases and injuries (for review, see Lowe, Scientific American (March/April 1996), p. 62). Due to this range of activities, IGF-1 has been tested in mammals for such widely disparate uses as wound healing, treatment of kidney disorders, treatment of diabetes, reversal of whole-body catabolic states such as AIDS-related wasting, treatment of heart conditions such as congestive heart failure, and treatment of neurological disorders (Guler *et al.*, Proc. Natl. Acad. Sci. USA, 85: 4889-4893 (1988); Schalch *et al.*, J. Clin. Metab., 77: 1563-1568 (1993); Froesch *et al.*, Horm. Res., 42: 66-71 (1994); Vlachopapadopoulou *et al.*, J. Clin. Endo. Metab., 12: 3715-3723 (1995); Saad *et al.*, Diabetologia, 37: Abstract 40 (1994); Schoenle *et al.*, Diabetologia, 34: 675-679 (1991); Morrow *et al.*, Diabetes, 42 (Suppl.): 269 (1993) (abstract); Kuzuya *et al.*, Diabetes, 42: 696-705 (1993); Schalch *et al.*, "Short-term metabolic effects of recombinant human insulin-like growth factor I (rhIGF-I) in type II diabetes mellitus", in: Spencer EM, ed., Modern Concepts of Insulin-like Growth Factors (New York: Elsevier: 1991) pp. 705-715; Zenobi *et al.*, J. Clin. Invest., 90: 2234-2241 (1993); Elahi *et al.*, "Hemodynamic and metabolic responses to human insulin-like growth factor-1 (IGF-I) in men," in: Modern Concepts of Insulin-Like Growth Factors, Spencer, EM, ed. (Elsevier: New York, 1991), pp. 219-224; Quinn *et al.*, New Engl. J. Med., 323: 1425-1426 (1990); Schalch *et al.*, "Short-term metabolic effects of recombinant human insulin-like growth factor I (rhIGF-I) in type II diabetes mellitus," in: Modern Concepts of Insulin-Like Growth Factors, Spencer, EM, ed., (Elsevier: New York, 1991), pp. 705-714; Schoenle *et al.*, Diabetologia, 34: 675-679 (1991); Usala *et al.*, N. Eng. J. Med., 327: 853-857 (1992); Lieberman *et al.*, J. Clin. Endo. Metab., 75: 30-36 (1992); Zenobi *et al.*, J. Clin. Invest., 90: 2234-2241 (1992); Zenobi *et al.*, J. Clin. Invest., 89: 1908-1913 (1992); Kerr *et al.*, J. Clin. Invest., 91: 141-147 (1993); Jabri *et al.*, Diabetes, 43: 369-374 (1994); Duerr *et al.*, J. Clin. Invest., 95: 619-627 (1995); Bondy, Ann Intern. Med., 120: 593-601 (1994); Hammerman and Miller, Am. J. Physiol., 265: F1-F14 (1993); Hammerman and Miller, J. Am. Soc. Nephrol., 5: 1-11 (1994); and Baringa *et al.*, Science, 264: 772-774 (1994)).

The patent literature also abounds with disclosures of various uses of IGF-1, or compounds that increase active concentration of IGF-I, to treat mammals, especially human patients, for example, U.S. Pat. Nos. 5,714,460; 5,273,961; 5,466,670; 5,126,324; 5,187,151; 5,202,119; 5,374,620; 5,106,832; 4,988,675; 5,106,832; 5,068,224; 5,093,317; 5,569,648; and 4,876,242; WO 92/11865; WO 96/01124; WO 91/03253; WO 93/25219; WO 93/08826; and WO 94/16722.

The IGF system is also composed of membrane-bound receptors for IGF-1, IGF-2, and insulin. The Type 1 IGF receptor (IGF-1R) is closely related to the insulin receptor in structure and shares some of its signaling pathways (Jones and Clemmons, *supra*). The IGF-2 receptor is a clearance receptor that appears not to transmit an intracellular signal (Jones and Clemmons, *supra*). Since IGF-1 and IGF-2 bind to IGF-1R with a much higher affinity than to the insulin receptor (Cascieri *et al.*, Biochemistry, 27: 3229-3233 (1988)), it is most likely that most of the effects of IGF-1 and IGF-2 are mediated by IGF-1R (Humbel, Eur. J Biochem. 190:445-462 (1990); Ballard *et al.*, "Does IGF-I ever act through the insulin receptor?", in Baxter *et al.* (Eds.), The Insulin-Like Growth Factors and Their Regulatory Proteins, (Amsterdam: Elsevier, 1994), pp. 131-138).

IGF-1R is an $\alpha 2\beta 2$ heterotetramer of disulfide-linked α and β subunits. $\alpha\beta$ dimers are themselves disulfide linked on the cell surface to form a covalent heterotetramer. As in the insulin/insulin receptor

complex, IGF-1 binds to the IGF-1R with a 1:2 stoichiometry (De Meyts, Diabetologia, 37: S135-S148 (1994)), with a high affinity site (K_d about 0.4 nM) and a low affinity site (K_d about 6 nM) (Tollefsen and Thompson, J. Biol. Chem., 263: 16267-16273 (1988)). The x-ray crystal structure of the first three domains of IGF-1R has been determined (Garrett *et al.*, Nature, 394, 395-399 (1998)). It contains three distinct domains (L1, Cys-rich, L2). Mutations that affect IGF-1 binding map to the concave surface of the receptor.

IGF-1R is a key factor in normal cell growth and development (Isaksson *et al.*, Endocrine Reviews, 8: 426-438 (1987); Daughaday and Rotwein, Endocrine Rev., 10:68-91 (1989)). Increasing evidence suggests, however, that IGF-lR signaling also plays a critical role in growth of tumor cells, cell transformation, and tumorigenesis (Baserga, Cancer Res., 55:249-252 (1995)). Key examples include loss of metastatic phenotype of murine carcinoma cells by treatment with antisense RNA to the IGF-lR (Long *et al.*, Cancer Res., 55:1006-1009 (1995)) and the *in vitro* inhibition of human melanoma cell motility (Stracke *et al.*, J Biol. Chem., 264:21554-21559 (1989)) and of human breast cancer cell growth by the addition of IGF-1R antibodies (Rohlik *et al.*, Biochem. Biophys. Res. Commun., 149:276- 281 (1987)).

The IGFs are potent breast cancer cell mitogens based on the observation that IGF-1 enhanced breast cancer cell proliferation *in vitro* (Cullen *et al.*, Cancer Res., 50:48-53 (1990)). Breast cancers express IGF-2 and IGF-lR, providing all the required effectors for an autocrine-loop-based proliferation paradigm (Quinn *et al.*, J Biol. Chem., 271:11477-11483 (1996); Steller *et al.*, Cancer Res., 56:1761-1765 (1996)). Because breast cancer is a common malignancy affecting approximately one in every eight women and is a leading cause of death from cancer in North American women (LeRoith *et al.*, Ann. Int. Med., 122:54-59 (1995)), new rational therapies are required for intervention. IGF-l can suppress apoptosis, and therefore cells lacking IGF-lRs or having compromised IGF-1R signaling pathways may give rise to tumor cells that selectively die via apoptosis (Long *et al.*, Cancer Res., 55:1006-1009 (1995)). Furthermore, it has recently become evident that alterations in IGF signaling in the context of other disease states, such as diabetes, may be responsible for exacerbating the complications of retinopathy (Smith *et al.*, Science, 276:1706-1709 (1997)) and nephropathy (Horney *et al.*, Am. J Physiol. 274: F1045-F1053 (1998)).

IGF-1 *in vivo* is mostly found in complex with a family of at least six serum proteins known as IGFBPs (Jones and Clemmons, *supra*; Bach and Rechler, Diabetes Reviews, 3: 38-61 (1995)), that modulate access of the IGFs to the IGF-1R. They also regulate the concentrations of IGF-1 and IGF-2 in the circulation and at the level of the tissue IGF-1R (Clemmons *et al.*, Anal. NY Acad. Sci. USA, 692:10-21 (1993)). The IGFBPs bind IGF-1 and IGF-2 with varying affinities and specificities (Jones and Clemmons, *supra*; Bach and Rechler, *supra*). For example, IGFBP-3 binds IGF-1 and IGF-2 with a similar affinity, whereas IGFBP-2 and IGFBP-6 bind IGF-2 with a much higher affinity than they bind IGF-1 (Bach and Rechler, *supra*; Oh *et al.*, Endocrinology, 132, 1337-1344 (1993)). The major carrier protein is IGFBP-3. Nothing is currently known about the stoichiometry of binding in these complexes of IGF-1 and its IGFBPs, due to the heterogeneous size of the complexes caused by glycosylation.

IGF-1 naturally occurs in human body fluids, for example, blood and human cerebral spinal fluid. Although IGF-1 is produced in many tissues, most circulating IGF-1 is believed to be synthesized in the liver. The IGFBPs are believed to modulate the biological activity of IGF-1 (Jones and Clemmons, *supra*), with IGFBP-1 (Lee *et al.*, Proc. Soc. Exp. Biol. & Med., 204: 4-29 (1993)) being implicated as the primary

binding protein involved in glucose metabolism (Baxter, "Physiological roles of IGF binding proteins", in: Spencer (Ed.), Modern Concepts of Insulin-like Growth Factors (Elsevier, New York, 1991), pp. 371-380). IGFBP-1 production by the liver is regulated by nutritional status, with insulin directly suppressing its production (Suikkari *et al.*, J. Clin. Endocrinol. Metab., 66: 266-272 (1988)).

The function of IGFBP-1 *in vivo* is poorly understood. The administration of purified human IGFBP-1 to rats has been shown to cause an acute, but small, increase in blood glucose (Lewitt *et al.*, Endocrinology, 129: 2254-2256 (1991)). The regulation of IGFBP-1 is somewhat better understood. It has been proposed (Lewitt and Baxter, Mol. Cell Endocrinology, 79: 147-152 (1991)) that when blood glucose rises and insulin is secreted, IGFBP-1 is suppressed, allowing a slow increase in "free" IGF-1 levels that might assist insulin action on glucose transport. Such a scenario places the function of IGFBP-1 as a direct regulator of blood glucose.

In most cases, addition of exogenous IGFBP blunts the effects of IGF-I. For example, the growth-stimulating effect of estradiol on the MCF-7 human breast cancer cells is associated with decreased IGFBP-3 mRNA and protein accumulation, while the anti-estrogen ICI 182780 causes growth inhibition and increased IGFBP-3 mRNA and protein levels (Huynh *et al.*, J Biol. Chem., 271:1016-1021 (1996); Oh *et al.*, Prog. Growth Factor Res., 6:503-512 (1995)). It has also been reported that the *in vitro* inhibition of breast cancer cell proliferation by retinoic acid may involve altered IGFBP secretion by tumor cells or decreased circulating IGF-1 levels *in vivo* (LeRoith *et al.*, Ann. Int. Med., 122:54-59 (1995); Oh *et al.*, (1995), *supra*). Contrary to this finding, treatment of MCF-7 cells with the anti-estrogen tamoxifen decreases IGF-IR signaling in a manner that is unrelated to decreased IGFBP production (Lee *et al.*, J Endocrinol., 152:39 (1997)). Additional support for the general anti-proliferative effects of the IGFBPs is the striking finding that IGFBP-3 is a target gene of the tumor suppressor, p53 (Buckbinder *et al.*, Nature, 377:646-649 (1995)). This suggests that the suppressor activity of p53 is, in part, mediated by IGFBP-3 production and the consequential blockade of IGF action (Buckbinder *et al.*, *supra*). These results indicate that the IGFBPs can block cell proliferation by modulating paracrine/autocrine processes regulated by IGF-1/IGF-2. A corollary to these observations is the finding that prostate-specific antigen (PSA) is an IGFBP-3-protease, which upon activation, increases the sensitivity of tumor cells to the actions of IGF-1/IGF-2 due to the proteolytic inactivation of IGFBP-3 (Cohen *et al.*, J. Endocr., 142:407-415 (1994)). The IGFBPs complex with IGF-1/IGF-2 and interfere with the access of IGF- I/IGF-2 to IGF-IRs (Clemmons *et al.*, Anal. NY Acad. Sci. USA, 692:10-21 (1993)). IGFBP-1, -2 and -3 inhibit cell growth following addition to cells *in vitro* (Lee *et al.* , J Endocrinol., 152:39 (1997); Feyen *et al.*, J Biol. Chem., 266:19469-19474 (1991)). Further, IGFBP-1 (McGuire *et al.*, J Natl. Cancer Inst., 84:1335-1341(1992); Figueroa *et al.*, J Cell Physiol., 157:229-236 (1993)), IGFBP-3 (Oh *et al.* (1995), *supra*; Pratt and Pollak, Biophys. Res. Commun., 198:292-297 (1994)) and IGFBP-2 have all been shown to inhibit IGF-1 or estrogen-induced breast cancer cell proliferation at nanomolar concentrations *in vitro*. These findings support the idea that the IGFBPs are potent antagonists of IGF action. There is also evidence for a direct effect of IGFBP-3 on cells through its own cell surface receptor, independent of IGF interactions (Oh *et al.*, J Biol. Chem., 268:14964-14971 (1993); Valentinis *et al.*, Mol. Endocrinol., 9:361-367 (1995)). Taken together, these findings underscore the importance of IGF and IGF-IR as targets for therapeutic use.

IGFs have mitogenic and anti-apoptotic influences on normal and transformed prostate epithelial cells (Hsing *et al.*, Cancer Research, 56: 5146 (1996); Culig *et al.*, Cancer Research, 54: 5474 (1994); Cohen *et al.*, Hormone and Metabolic Research, 26: 81 (1994); Iwamura *et al.*, Prostate, 22: 243 (1993); Cohen *et al.*, J. Clin. Endocrin. & Metabol., 73: 401 (1991); Rajah *et al.*, J. Biol. Chem., 272: 12181 (1997)). Most circulating IGF-1 originates in the liver, but IGF bioactivity in tissues is related not only to levels of circulating IGFs and IGFBPs, but also to local production of IGFs, IGFBPs, and IGFBP proteases (Jones and Clemmons, *supra*). Person-to-person variability in levels of circulating IGF-1 and IGFBP-3 (the major circulating IGFBP (Jones and Clemmons, *supra*)) is considerable (Juul *et al.*, J. Clin. Endocrinol. & Metabol., 78: 744 (1994); Juul *et al.*, J. Clin. Endocrinol. & Metabol., 80: 2534 (1995)), and heterogeneity in serum IGF-1 levels appears to reflect heterogeneity in tissue IGF bioactivity. Markers relating to IGF-axis components can be used as a risk marker for prostate cancer, as PSA is likewise used (WO 99/38011).

Unlike most other growth factors, the IGFs are present in high concentrations in the circulation, but only a small fraction of the IGFs is not protein bound. For example, it is generally known that in humans or rodents, less than 1% of the IGFs in blood is in a "free" or unbound form (Juul *et al.*, Clin. Endocrinol., 44: 515-523 (1996); Hizuka *et al.*, Growth Regulation, 1: 51-55 (1991); Hasegawa *et al.*, J. Clin. Endocrinol. Metab., 80: 3284-3286 (1995)). The overwhelming majority of the IGFs in blood circulate as part of a non-covalently associated ternary complex composed of IGF-1 or IGF-2, IGFBP-3, and a large protein termed the acid-labile subunit (ALS). The ternary complex of an IGF, IGFBP-3, and ALS has a molecular weight of approximately 150,000 daltons, and it has been suggested that the function of this complex in the circulation may be to serve as a reservoir and buffer for IGF-1 and IGF-2, preventing rapid changes in free IGF-1 or IGF-2.

There has been much work identifying the regions on IGF-1 and IGF-2 that bind to the IGFBPs (Bayne *et al.*, J. Biol. Chem., 265: 15648-15652 (1990); Dubaquie and Lowman, Biochemistry, 38: 6386-6396 (1999); and U.S. Pat. Nos. 5,077,276; 5,164,370; and 5,470,828). For example, it has been discovered that the N-terminal region of IGF-1 and IGF-2 is critical for binding to the IGFBPs (U.S. Pat. Nos. 5,077,276; 5,164,370; and 5,470,828). Thus, the natural IGF-I variant, designated des (1-3) IGF-1, binds poorly to IGFBPs.

A similar amount of research has been devoted to identifying the regions on IGF-1 and IGF-2 that bind to IGF-1R (Bayne *et al.*, *supra*; Oh *et al.*, Endocrinology (1993), *supra*). It was found that the tyrosine residues in IGF-1 at positions 24, 31, and 60 are crucial to the binding of IGF-1 to IGF-1R (Bayne *et al.*, *supra*). Mutant IGF-1 molecules where one or more of these tyrosine residues are substituted showed progressively reduced binding to IGF-1R. Bayne *et al.*, *supra*, also investigated whether such mutants of IGF-1 could bind to IGF-1R and to the IGFBPs. They found that quite different residues on IGF-1 and IGF-2 are used to bind to the IGFBPs from those used to bind to IGF-1R. It is therefore possible to produce IGF variants that show reduced binding to the IGFBPs, but, because they bind well to IGF-1R, show maintained activity in *in vitro* activity assays.

Also reported was an IGF variant that binds to IGFBPs but not to IGF receptors and therefore shows reduced activity in *in vitro* activity assays (Bar *et al.*, Endocrinology, 127: 3243-3245 (1990)). In this variant, designated (1-27,gly^4, 38-70)-hIGF-1, residues 28-37 of the C-region of human IGF-I are replaced by a four-residue glycine bridge.

Other truncated IGF-1 variants are disclosed. For example, in the patent literature, WO 96/33216 describes a truncated variant having residues 1-69 of authentic IGF-1. EP 742,228 discloses two-chain IGF-1 superagonists, which are derivatives of the naturally occurring, single-chain IGF-1 having an abbreviated C-region. The IGF-1 analogs are of the formula: BC^nA wherein B is the B-region of IGF-1 or a functional analog thereof, C is the C-region of IGF-1 or a functional analog thereof, n is the number of amino acids in the C-region and is from about 6 to about 12, and A is the A-region of IGF-1 or a functional analog thereof.

Additionally, Cascieri *et al.*, Biochemistry, 27: 3229-3233 (1988) discloses four mutants of IGF-1, three of which have reduced affinity to IGF-1R. These mutants are: (Phe^{23},Phe^{24},Tyr^{25})IGF-1 (which is equipotent to human IGF-1 in its affinity to the Types 1 and 2 IGF and insulin receptors), (Leu^{24})IGF-1 and (Ser^{24})IGF-1 (which have a lower affinity than IGF-1 to the human placental IGF-1R, the placental insulin receptor, and the IGF-1R of rat and mouse cells), and desoctapeptide (Leu^{24})IGF-1 (in which the loss of aromaticity at position 24 is combined with the deletion of the carboxyl-terminal D-region of hIGF-1, which has lower affinity than (Leu^{24})IGF-1 for the IGF-1R and higher affinity for the insulin receptor). These four mutants have normal affinities for human serum binding proteins.

Bayne *et al.*, J. Biol. Chem., 263: 6233-6239 (1988) discloses four structural analogs of human IGF-1: a B-chain mutant in which the first 16 amino acids of IGF-1 were replaced with the first 17 amino acids of the B-chain of insulin, (Gln^3,Ala^4)IGF-1, (Tyr^{15},Leu^{16})IGF-1, and (Gln^3,Ala^4,Tyr^{15},Leu^{16})IGF-1. These studies identify some of the regions of IGF-1 that are responsible for maintaining high-affinity binding with the serum binding protein and the Type 2 IGF receptor.

In another study, Bayne *et al.*, J. Biol. Chem., 264: 11004-11008 (1988) discloses three structural analogs of IGF-1: (1-62)IGF-1, which lacks the carboxyl-terminal 8-amino-acid D-region of IGF-1; (1-27,Gly^4,38-70)IGF-1, in which residues 28-37 of the C-region of IGF-1 are replaced by a four-residue glycine bridge; and (1-27,Gly^4,38-62)IGF-1, with a C-region glycine replacement and a D-region deletion. Peterkofsky *et al.*, Endocrinology, 128: 1769-1779 (1991) discloses data using the Gly^4 mutant of Bayne *et al.*, *supra* (vol. 264).

Cascieri *et al.*, J. Biol. Chem., 264: 2199-2202 (1989) discloses three IGF-1 analogs in which specific residues in the A-region of IGF-1 are replaced with the corresponding residues in the A chain of insulin. The analogs are: (Ile^{41},Glu^{45},Gln^{46},Thr^{49},Ser^{50},Ile^{51},Ser^{53},Tyr^{55},Gln^{56})IGF-1, an A-chain mutant in which residue 41 is changed from threonine to isoleucine and residues 42-56 of the A-region are replaced; (Thr^{49},Ser^{50},Ile^{51})IGF-1; and (Tyr^{55},Gln^{56})IGF-1.

Clemmons *et al.*, J. Biol. Chem., 265: 12210-12216 (1990) discloses use of IGF-1 analogs that have reduced binding affinity for either IGF-1R or binding proteins to study the ligand specificity of IGFBP-1 and the role of IGFBP-1 in modulating the biological activity of IGF-1.

WO 94/04569 discloses a specific binding molecule, other than a natural IGFBP, that is capable of binding to IGF-1 and can enhance the biological activity of IGF-1.

Peptides that bind to IGFBP-1, block IGF-1 binding to this binding protein, and thereby release "free-IGF" activity from mixtures of IGF-1 and IGFBP-1 have been recently described (Lowman *et al.*, Biochemistry, 37: 8870-8878 (1998); WO 98/45427 published October 15, 1998; Lowman *et al.*,

International Pediatric Nephrology Association, Fifth Symposium on Growth and Development in Children with Chronic Renal Failure (New York, March 13, 1999)). Also described is the natural molecule, des(1-3)IGF-1, which shows selectively reduced affinity for some of the IGF binding proteins, yet a maintained affinity for the IGF receptor (U.S. Pat. Nos. 5,077,276; 5,164,370; 5,470,828).

Exploitation of the interaction between IGF and IGFBP in screening, preventing, or treating disease has been limited, however, because of a lack of specific antagonists. To date, only one publication is known to exist that describes the application of an IGF-I/ IGF-2 antagonist as a potential therapeutic adjunct in the treatment of cancer (Pietrzkowski *et al.*, Cancer Res., 52: 6447-6451 (1992)). In that report, a peptide corresponding to the D-region of IGF- I was synthesized for use as an IGF-1/2 antagonist. This peptide exhibited questionable inhibitory activity against IGF-I. The basis for the observed inhibition is unclear, as the D-region does not play a significant role in IGF-I R binding but rather, in IGF-1 binding to the insulin receptor (Cooke *et al.*, Biochem., 30:5484-5491 (1991); Bayne *et al.*, *supra* (Vol. 264); Yee *et al.*, Cell Growth and Different., 5:73-77 (1994)).

WO 00/23469 discloses the portions of IGFBP and IGF peptides that account for IGF-IGFBP binding, i.e., an isolated IGF binding domain of an IGFBP or modification thereof that binds IGF with at least about the same binding affinity as the full-length IGFBP. The patent publication also discloses an IGF antagonist that reduces binding of IGF to an IGF receptor, and/or binds to a binding domain of IGFBP.

Additionally, EP 639981 discloses pharmaceutical compositions comprising short peptides that function as IGF-1 receptor antagonists. The peptides used in the pharmaceutical compositions consist of less than 25 amino acids, comprise at least a portion of the C- or D-region from IGF-1, and inhibit IGF-1-induced autophosphorylation of IGF-1 receptors.

Polypeptides, including the IGF molecules, have a three-dimensional structure determined by the primary amino acid sequence and the environment surrounding the polypeptide. This three-dimensional structure establishes the activity, stability, binding affinity, binding specificity, and other biochemical attributes of the polypeptide. Thus, knowledge of the three-dimensional structure of a protein can provide much guidance in designing agents that mimic, inhibit, or improve its biological activity in soluble or membrane-bound forms.

The three-dimensional structure of a polypeptide may be determined in a number of ways. Many of the most precise methods employ x-ray crystallography (Van Holde, Physical Biochemistry (Prentice Hall: N.J., 1971), pp. 221-239). This technique relies on the ability of crystalline lattices to diffract x-ray or other forms of radiation. Diffraction experiments suitable for determining the three-dimensional structure of macromolecules typically require high-quality crystals. Unfortunately, such crystals have been unavailable for IGF-1 as well as many other proteins of interest. Crystals have been described for M-CSF (EP 668,914B1), CD40 ligand (WO 97/00895), and a BC2 Fab fragment (WO 99/01476), for example.

The crystallization of insulin is an intensively researched field, both with respect to work on structural analysis (Adams *et al.*, Nature, 224: 491 (1969)) and pharmaceutical applications. Examples of insulin crystal suspensions that are used therapeutically include suspensions of rhombohedral zinc-insulin crystals that are stable in the presence of 0.8 to 2.5% of zinc (based on the weight of insulin) at a neutral pH value and exhibit a delayed action, and isophane insulin protamine crystals, which are used in delayed action products in the form of small rods. A few other crystal modifications of insulin are furthermore

known, but these have hitherto been of interest only for X-ray structure analysis. Thus, zinc-free orthorhombic and monoclinic crystals have been obtained under acid pH conditions (Einstein and Low, Acta Crystallogr., 15: 32-34 (1962)). Smaller rhombic dodecahedra, which are to be classified in the cubic space group, have been obtained at the isoelectric point, also in the absence of zinc. Finally, a monoclinic crystal form of insulin has been obtained above the isoelectric point in the presence of zinc and in the presence of phenol or phenol derivatives. These crystals grow to a considerable size (up to 3 mm) within a few days and have sharp edges. Interestingly, these crystals have been found only on glass surfaces and not on the free surface of the solution. Crystal suspensions and other crystal forms of insulin preparations and insulin analogs are described, for example, in such representative patents as U.S. Pat. Nos. 4,959,351; 5,840,680; 5,834,422; 6,127,334; 5,952,297; 5,650,486; 5,898,028; 5,898,067; 5,948,751; 5,747,642; 5,597,893; 5,547,930; 5,534,488; 5,504,188; 5,461,031; and 5,028,587.

Various methods for preparing crystalline proteins and polypeptides are known in the art (McPherson *et al.*, "Preparation and Analysis of Protein Crystals," McPherson (Robert E. Krieger Publishing Company, Malabar, FL, 1989); Weber, Advances in Protein Chemistry, 41: 1-36 (1991); US Pat Nos. 4,672,108 and 4,833,233). Although there are multiple approaches to crystallizing polypeptides, no single set of conditions provides a reasonable expectation of success, especially when the crystals must be suitable for x-ray diffraction studies. Significant effort is required to obtain crystals of sufficient size and resolution to provide accurate information regarding the structure. For example, once a protein of sufficient purity is obtained, it must be crystallized to a size and clarity that is useful for x-ray diffraction and subsequent structure resolution. Further, although the amino acid sequence of a target protein may be known, this sequence information does not allow an accurate prediction of the crystal structure of the protein. Nor does the sequence information afford an understanding of the structural, conformational, and chemical interactions between a ligand such as an IGFBP and its protein target. Thus, although crystal structures can provide a wealth of valuable information in the field of drug design and discovery, crystals of certain biologically relevant compounds such as IGF-1 are not readily available to those skilled in the art. High-quality, diffracting crystals of IGF-1 would assist the determination of its three-dimensional structure.

Generation of specific IGF-1 antagonists has been restricted, at least in part, because of difficulties in studying the structure of IGF and IGFBPs. Due to the inability to obtain crystals of IGF-1 suitable for diffraction studies, for example, an extrapolation of IGF-1 structure based on the crystal structure of porcine insulin was the most important structural road map for IGF-1 available (Blundell *et al.* , Proc. Natl. Acad. Sci. USA , 75:180- 184 (1978)). See also Blundell *et al.*, Fed. Proc., 42: 2592-2597 (1983), which discloses tertiary structures, receptor binding, and antigenicity of IGFs. Based on studies of chemically modified and mutated IGF-1, a number of common residues between IGF- I and insulin have been identified as being part of the IGF-IR-insulin receptor contact site, in particular, the aromatic residues at positions 23-25.

Using NMR and restrained molecular dynamics, the solution structure of IGF-1 was recently reported (Cooke *et al.*, *supra*). The resulting minimized structure was shown to better fit the experimental findings on modified IGF-1, as well as the extrapolations made from the structure-activity studies of insulin. Further, De Wolf *et al.*, Protein Sci., 5: 2193-2202 (1996) discloses the solution structure of a mini-IGF-1. Sato *et al.*, Int. J. Pept. Protein Res., 41: 433-440 (1993) discloses the three-dimensional structure of IGF-1 determined by 1H-NMR and distance geometry. Laajoki *et al.*, J. Biol. Chem., 275: 10009-10015 (2000)

discloses the solution structure and backbone dynamics of long-[Arg(3)]IGF-1. See also Laajoki *et al.*, FEBS Lett., 420: 97-102 (1997)). The small number of NMR models available for IGF-1 are not very well defined, as there are large RMSDs between the backbone atoms of the helical segments. The best NMR model is of IGF-2 in which three alpha-helices are shown. See Torres *et al.*, J. Mol. Biol., 248: 385-401 (1995), which discloses the solution structure of human IGF-2 and its relationship to receptor and binding protein interactions. In all structures, the C- and D-regions are very poorly defined.

In addition to providing structural information, crystalline polypeptides provide other advantages. For example, the crystallization process itself further purifies the polypeptide and satisfies one of the classical criteria for homogeneity. In fact, crystallization frequently provides unparalleled purification quality, removing impurities that are not removed by other purification methods such as HPLC, dialysis, conventional column chromatography, etc. Moreover, crystalline polypeptides are often stable at ambient temperatures and free of protease contamination and other degradation associated with solution storage. Crystalline polypeptides may also be useful as pharmaceutical preparations. Finally, crystallization techniques in general are largely free of problems such as denaturation associated with other stabilization methods (e.g. lyophilization). Thus, there exists a significant need for preparing IGF-1 compositions in crystalline form and determining their three-dimensional structure. The present invention fulfills this and other needs. Once crystallization has been accomplished, crystallographic data provides useful structural information that may assist the design of peptides that may serve as agonists or antagonists. In addition, the crystal structure provides information useful to map the receptor-binding domain, which could then be mimicked by a small non-peptide molecule that may serve as an antagonist or agonist. Also, findings regarding the detergent's inhibition of the binding of IGFBP to IGF-1 can be used to identify new IGF-1 agonists.

Summary of the Invention

Accordingly, the invention is as claimed. IGF-1 has been crystallized and its structure determined using multiwavelength anomalous diffraction (MAD) at 1.8 angstroms resolution by exploiting the anomalous scattering of a single bromide ion and six of the seven sulfur atoms of IGF-1. The C-region of IGF-1, which is ordered in the crystal structure, forms a type II beta-turn and mediates a crystal packing interaction across a crystallographic dyad. The solution state of IGF-1 was characterized by analytical ultracentrifugation, and the results indicate that IGF-1 exists primarily as a monomer at neutral pH, with only a slight tendency to dimerize at millimolar concentrations. A molecule of detergent, N, N-bis(3-D-gluconamidopropyl)-deoxycholamine (deoxy big CHAPS), mediates a crystal packing contact between symmetry-related molecules. Biophysical and biochemical data show that the N, N-bis(3-D-gluconamidopropyl)-deoxycholamine binds to IGF-1 specifically and blocks binding of IGFBP-1 and IGFBP-3.

Accordingly, in one aspect, the invention provides a crystal formed by IGF-1 that diffracts x-ray radiation to produce a diffraction pattern representing the three-dimensional structure of the IGF-1. Preferably this crystal has approximately the following cell constants a=31.831 Å, b=71.055 Å, c=65.995 Å, and a space group of $C222_1$. Also preferably, the IGF-1 contains an A-, B-, C-, and D-region and forms a

dimer in the crystal, and further preferred is the crystal comprising a receptor binding site at the dimer interface.

The invention also provides a composition comprising the above crystal. Preferably in this composition the IGF-1 is biologically active when resolubilized. The invention further provides a method of treating a mammal suffering from an agonist disorder, preferably a human patient, said method comprising administering to said mammal an effective amount of the above resolubilized composition.

The invention also provides a method of crystallizing IGF-1 comprising the steps of:

(a) mixing an aqueous solution comprising IGF-1 with a reservoir solution comprising a precipitant to form a mixed volume; and

(b) crystallizing the mixed volume.

The invention also provides crystalline IGF-1 produced by the above method.

Additionally, the invention provides a method for determining a three-dimensional structure of IGF-1 comprising:

(a) crystallizing the IGF-1;

(b) irradiating the crystalline IGF-1 to obtain a diffraction pattern characteristic of the crystalline IGF-1; and

(c) transforming the diffraction pattern into the three-dimensional structure of the IGF-1.

Further, the invention provides a machine-readable data storage medium comprising a data storage material encoded with machine-readable data that, when read by an appropriate machine, displays a three-dimensional representation of a crystal of a molecule comprising IGF-1.

In further aspects, the invention provides an IGF-1 crystal with the structural coordinates shown in Appendix 1.

Additionally, the invention provides a method of using a three-dimensional structure of IGF-1 derived from an IGF-1 crystal wherein the three-dimensional structure of IGF-1 includes an IGF-1 receptor-binding region, the method comprising identifying compounds having structures that interact with the receptor-binding region of the three-dimensional structure of IGF-1 and function as an IGF-1 agonist or antagonist. Preferably in such method the three-dimensional structure of IGF-1 includes alpha-carbon coordinates substantially the same as those of the structural information presented in Appendix 1.

In another aspect, the invention provides a method of identifying IGF-1 agonists or antagonists comprising the steps of:

(a) crystallizing IGF-1 to form IGF-1 crystals, the IGF-1 crystals containing a group of amino acid residues defining an IGF-1 receptor-binding region;

(b) irradiating the IGF-1 crystals from step (a) to obtain a diffraction pattern of the IGF-1 crystals;

(c) determining a three-dimensional structure of IGF-1 from the diffraction pattern, the structure including an IGF-1 receptor-binding region; and

(d) identifying an IGF-1 agonist or antagonist having a three-dimensional structure that functionally duplicates essential IGF receptor-binding, solvent-accessible residues presenting the three-dimensional structure of the IGF-1 receptor-binding region, said IGF-1 agonist or antagonist having altered signal transduction capacity to IGF-1-responsive cells, as compared to IGF-1.

Preferably, in this method the solvent-accessible residues do not participate in formation of the IGF-1 interface.

According to certain further aspects, the invention includes a method of designing a compound, such as a peptidomimetic, that mimics the 3-dimensional surface structure of IGF-1 comprising the steps of:

(a) determining the 3-dimensional structure of the IGF-1; and

(b) designing a compound that mimics the 3-dimensional surface structure of the IGF-1.

According to a further embodiment, the invention provides a method for identifying a peptidomimetic that binds IGF-1 and blocks binding of an IGFBP or a receptor that binds to IGF-1 comprising the steps of:

(a) searching a molecular structure database with the structural parameters or structural coordinates provided in Appendix 1; and

(b) selecting a molecule from the database that mimics the structural parameters or structural coordinates of the IGF-1.

The invention also provides a method for determining at least a portion of a three-dimensional structure of a molecular complex, said complex comprising IGF-1 and said method comprising the steps of:

(a) determining the structural coordinates of a crystal of IGF-1;

(b) calculating phases from the structural coordinates;

(c) calculating an electron density map from the phases obtained in step (b); and

(d) determining the structure of at least a portion of the complex based on said electron density map.

Preferably the structural coordinates used in step (a) are substantially the same as those described in Appendix 1 or describe substantially the same crystal as the coordinates in Appendix 1.

The invention also provides a method for evaluating the ability of a chemical entity to associate with IGF-1 or a complex thereof, the method comprising the steps of:

(a) employing computational or experimental means to perform a fitting operation between the chemical entity and the IGF-1 or complex thereof, thereby obtaining data related to the association; and

(b) analyzing the data obtained in step (a) to determine the characteristics of the association between the chemical entity and the IGF-1 or complex thereof.

The invention also provides a chemical entity identified by the above method that interferes with the *in vivo* or *in vitro* association between IGF-1 and its receptor or between IGF-1 and at least one of its binding proteins, or associates with a binding site on IGF-1.

Also provided is a heavy-atom derivative of a crystallized form of IGF-1.

The invention also comprises a method of computationally or experimentally evaluating a chemical entity to obtain information about its association with one or more binding sites of IGF-1 using a crystal of IGF-1 having the structural coordinates described in Appendix 1.

Any peptide analogs and other chemical entities identified using the above methods of the present invention are useful in the therapeutic methods described herein and as pharmaceutical compositions.

The invention also provides a method of identifying indirect agonists of IGF-1 comprising the steps of:

(a) comparing the ability of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine to inhibit binding of IGFBP-1 or IGFBP-3 to IGF-1 with the ability of a candidate indirect agonist of IGF-1 to so inhibit binding; and

(b) determining whether the candidate agonist inhibits such binding at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

In a preferred embodiment, the comparison is accomplished by competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and the candidate agonist. In a more preferred embodiment, inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholamine or the candidate agonist with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based ELISA assay.

The invention further provides a method of identifying indirect agonists of IGF-1 comprising co-crystallizing a candidate indirect agonist of IGF-1 with IGF-1 to form a co-crystalline structure and determining if the candidate agonist binds to one or both of two patches on IGF-1, wherein one patch has the amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and the second patch has the amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55, and wherein binding occurs if there is at least one contact between each listed amino acid residue of a given patch and the candidate agonist that is less than or equal to 6 angstroms in the co-crystalline structure. In preferred embodiments, the candidate agonist inhibits binding of IGFBP-1 or -3 to IGF-1 at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. More preferred is the method wherein inhibition of binding is measured using a competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and the candidate agonist. Most preferred is the method wherein inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholamine or the candidate agonist with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based ELISA assay.

Also provided herein is a method for treating an IGF-1 agonist disorder in a mammal comprising administering to the mammal an effective amount of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

Further provided herein is a co-crystalline complex of IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

Brief Description of the Drawings

Figure 1 aligns the sequences of IGF-1 (SEQ ID NO:1), IGF-2 (SEQ ID NO:2), and insulin (SEQ ID NO:3). The A-, B-, and C-chains of insulin (and the sequences of IGF-1 and IGF-2 corresponding thereto) are shown respectively in bold, underlined, and italicized text. The three aromatic residues are shown by outlining the text. The residues marked with a (!) have been demonstrated to be important for binding to the IGF-1 receptor. The residues marked with a "*" have been shown to be important for binding to IGFBP-1 and IGFBP-3. The carboxyl-terminal residues comprising the D-region of IGF-1 and IGF-2 are depicted in regular type.

Figure 2 is a ribbon diagram of IGF-1 showing the backbone fold. In the Ramachandran plot, 97.7% is most favored and 2.3% is allowed.

Figure 3 is a ribbon diagram of both IGF-1 (left structure) and insulin (right structure).

Figure 4 is a ribbon diagram of IGF-1 showing that the detergent used in the reservoir solution, (N, N-bis(3-D-gluconamidopropyl)-deoxycholamine), binds into a small hydrophilic cleft at the base of the B-helix. The detergent is represented by lighter gray structures than the IGF-1 structures.

Figure 5 is a ribbon diagram of IGF-1 as a dimer, with the detergent shown in lighter gray.

Figure 6 is a ribbon diagram of IGF-1 as a dimer, showing that the residues important for receptor binding (indicated by ring structures in the center portion of the figure) cluster at the dimer interface. The detergent is shown in lighter gray at the outer portions of the figure.

Figures 7A and 7B are ribbon diagrams of IGF-1 demonstrating, as does Fig. 4, that the detergent used in the reservoir solution (N, N-bis(3-D-gluconamidopropyl)-deoxycholamine), shown in stick form, binds into a small hydrophilic cleft at the base of the B-helix. In Fig. 7A the detergent head group is inserted into the cleft lined by residues Leu 5, Phe 16, Val 17, Leu 54, and Leu 57. The various shades of gray are according to the alanine-scanning mutagenesis results of Dubaquie and Lowman, *supra*, with the Phe 16, Val 17, and Leu 5 regions indicating a 5-10 fold reduction, the Glu 3 region a 10-100 fold reduction, and the Pro 63 and Pro 63' regions a >100 fold reduction in affinity for IGFBP-1. The black part at the far right corresponds to the symmetry-related IGF-1 molecule that forms the crystallographic dimer. The circle near Leu 54 indicates the C10 atom of the detergent, which differs from another detergent (3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate; or CHAPS) by having a hydroxyl group at this position. Fig. 7B shows the view from the opposite surface of the detergent and depicts the interactions of the detergent molecule with a symmetry-related IGF-1 molecule. As in Fig. 7A the various shades of gray are according to the alanine-scanning mutagenesis results of Dubaquie and Lowman, *supra*, with the group near Gln 15 indicating a 5-10 fold reduction, the far left medium gray molecules, the Leu 10 region molecules, and the far right medium gray region indicating a 10-100 fold reduction, and the black regions at Phe 49 and Gly 7 indicating a >100 fold reduction in affinity for IGFBP-1. The black regions to the right of the detergent molecule correspond to the symmetry-related IGF-1 molecule that forms the crystallographic dimer. The circle near Gln 15 indicates the C10 atom of the detergent, as noted above for Fig. 7A. This figure was prepared using the program INSIGHT (MSI, San Diego, CA).

Figure 8 shows a graph resulting from a detergent/IGFBP competition binding study. In this experiment, N, N-bis(3-D-gluconamidopropyl)-deoxycholamine was used as a competitive inhibitor of IGF-1 binding to immobilized IGFBP-1 (open circles) or IGFBP-3 (open squares). As a positive control, soluble IGFBP-1 (solid circles) or IGFBP-3 (solid squares) was used as a competitive inhibitor of IGF-1 binding to immobilized IGFBP-1 or IGFBP-3, respectively. Each data point represents the average of three independent experiments.

Figure 9A shows a non-linear least-squares analysis of sedimentation equilibrium data for IGF-1 in solution. Data collected at rotor speeds of 30,000 rpm (open triangles) and 35,000 rpm (open squares) were fit as an ideal monomer-dimer self-association model. The solid lines are the fits of the data. Figure 9B shows the residuals plotted for both rotor speeds after accounting for the data by the fitting procedure. They are randomly distributed around zero, indicating that the monomer-dimer model is correct for this interaction.

Figure 10A shows a ribbon diagram determined by NMR of a complex of IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine), and Figure 10B shows a ribbon diagram determined by NMR of a complex of IGF-1 bound to a phage-derived IGF-1 antagonist peptide designated IGF-F1-1.

Description of the Preferred Embodiments

A. Definitions

As used herein, "IGF-1" refers to human insulin-like growth factor-1 unless otherwise noted, and has the human native mature IGF-1 sequence without a N-terminal methionine, as described, for example, by EP 230,869 published August 5, 1987; EP 128,733 published December 19, 1984; or EP 288,451 published October 26, 1988.

An "IGFBP" or an "IGF binding protein" refers to a protein or polypeptide normally associated with or bound or complexed to IGF-1, whether or not it is circulatory (*i.e.*, in serum or tissue). This definition includes IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, Mac 25 (IGFBP-7), and prostacyclin-stimulating factor (PSF) or endothelial cell-specific molecule (ESM-1), as well as other proteins with high homology to IGFBPs. Mac 25 is described, for example, in Swisshelm *et al.*, Proc. Natl. Acad. Sci. USA, 92: 4472-4476 (1995) and Oh *et al.*, J. Biol. Chem., 271: 30322-30325 (1996). PSF is described in Yamauchi *et al.*, Biochemical Journal, 303: 591-598 (1994). ESM-1 is described in Lassalle *et al.*, J. Biol. Chem., 271: 20458-20464 (1996). For other identified IGFBPs, see, *e.g.*, EP 375,438 published 27 June 1990; EP 369,943 published 23 May 1990; U.S. Pat. No. 5,258,287; WO 89/09268 published 5 October 1989; Wood *et al.*, Molecular Endocrinology, 2: 1176-1185 (1988); Brinkman *et al.*, The EMBO J., 7: 2417-2423 (1988); Lee *et al.*, Mol. Endocrinol., 2: 404-411 (1988); Brewer *et al.*, BBRC, 152: 1289-1297 (1988); EP 294,021 published 7 December 1988; Baxter *et al.*, BBRC, 147: 408-415 (1987); Leung *et al.*, Nature, 330: 537-543 (1987); Martin *et al.*, J. Biol. Chem., 261: 8754-8760 (1986); Baxter *et al.*, Comp. Biochem. Physiol., 91B: 229-235 (1988); WO 89/08667 published 21 September 1989; WO 89/09792 published 19 October 1989; and Binkert *et al.*, EMBO J., 8: 2497-2502 (1989). IGFBP-1 and IGFBP-3 bind to different residues of IGF-1.

As used herein, "human IGF-1 receptor" or just "IGF-1 receptor" refers to any receptor for IGF-1 found in humans and includes the Type 1 and Type 2 IGF receptors in humans to which human IGF-1 binds, such as the placental IGF-1R, *etc.*

An "indirect agonist of IGF-1" is a molecule that releases IGF-1 *in situ* from IGFBP-3 or IGFBP-1 so that the IGF-1 released is active and interacts with its receptor.

"Peptides" are molecules having at least two amino acids and include polypeptides having at least about 60 amino acids. Preferably, the peptides have about 10 to about 60 amino acids, more preferably about 10-25, and most preferably about 12-25 amino acids. The definition includes linear and cyclic peptides, peptide derivatives, their salts, or optical isomers.

As used herein, "mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic, and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, sheep, pigs, cows, *etc.* The preferred mammal herein is a human. The term "non-adult" refers to mammals that are from perinatal age (such as low-birth-weight infants) up to the age of puberty, the latter being those that have not yet reached full growth potential.

As used herein, the term "treating" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those prone to having the disorder or diagnosed with the disorder or those in which the disorder is to be prevented.

A "disorder" is any condition that would benefit from treatment with an IGF-1 agonist ("agonist disorder") or antagonist ("antagonist disorder"). This includes chronic and acute disorders or diseases including those pathological conditions that predispose the mammal to the disorder in question. The disorder being treated may be a combination of two or more of the agonist or antagonist disorders listed below.

Non-limiting examples of antagonist disorders include benign and malignant tumors, leukemias and lymphoid malignancies, neuronal, glial, astrocytal, hypothalamic and other glandular, macrophagal, epithelial, stromal and blastocoelic disorders, and inflammatory, angiogenic and immunologic disorders, diabetic complications such as diabetic retinopathies or neuropathies, age-related macular degeneration, ophthalmic surgery such as cataract extraction, a corneal transplant, glaucoma filtration surgery and keratoplasty, surgery to correct refraction, i.e., a radial keratotomy, also in sclera macular holes and degeneration, retinal tears, vitreoretinopathy, miscellaneous disorders, cataract disorders of the cornea such as the sequelae of radial keratotomy, dry eye, viral conjunctivitis, ulcerative conjunctivitis, wounds such as corneal epithelial wounds, Sjogren's syndrome, retinal disorders such as macular and retinal edema, vision-limited scarring, retinal ischemia, and proliferative vitreous retinopathy.

More preferably, such antagonist disorders include diabetic complications exacerbated by IGF-1, ischemic injury, and diseases associated with undesirable cell proliferation such as cancer, restenosis, and asthma. If the disorder is a diabetic complication exacerbated by IGF-1, such complication can include diabetic retinopathy or diabetic nephropathy. The efficacy of the treatment can be evidenced by a reduction in clinical manifestations or symptoms, including, for example, improved renal clearance, improved vision, or a reduction in the amount of IGF-1 available for binding to an IGF-1 receptor. If the disorder is an ischemic injury, it can include strokes, myocardial ischemia, and ischemic injury to the kidneys.

Examples of agonist disorders for purposes herein include any condition that would benefit from treatment with an IGF-1, including but not limited to, for example, lung diseases, hyperglycemic disorders as set forth below, renal disorders, such as acute and chronic renal insufficiency, end-stage chronic renal failure, glomerulonephritis, interstitial nephritis, pyelonephritis, glomerulosclerosis, *e.g.,* Kimmelstiel-Wilson in diabetic patients and kidney failure after kidney transplantation, obesity, GH-insufficiency, Turner's syndrome, Laron's syndrome, short stature, undesirable symptoms associated with aging such as obesity and increased fat mass-to-lean ratios, immunological disorders such as immunodeficiencies including decreased CD4 counts and decreased immune tolerance or chemotherapy-induced tissue damage, bone marrow transplantation, diseases or insufficiencies of cardiac structure or function such as heart dysfunctions and congestive heart failure, neuronal, neurological, or neuromuscular disorders, *e.g.,* peripheral neuropathy, multiple sclerosis, muscular dystrophy, or myotonic dystrophy, and catabolic states associated with wasting caused by any condition, including, *e.g.,* trauma or wounding, or infection such as with a bacterium or human virus such as HIV, wounds, skin disorders, gut structure and function that need restoration, and so forth. The preferred agonist disorders targeted for treatment herein are diabetes and

obesity, heart dysfunctions, AIDS-related wasting, kidney disorders, neurological disorders, whole body growth disorders, and immunological disorders.

As used herein, the term "hyperglycemic disorders" refers to all forms of diabetes and disorders resulting from insulin resistance, such as Type I and Type II diabetes, as well as severe insulin resistance, hyperinsulinemia, and hyperlipidemia, *e.g.*, obese subjects, and insulin-resistant diabetes, such as Mendenhall's Syndrome, Werner Syndrome, leprechaunism, lipoatrophic diabetes, and other lipoatrophies. The preferred hyperglycemic disorder is diabetes, especially Type 1 and Type II diabetes. "Diabetes" itself refers to a progressive disease of carbohydrate metabolism involving inadequate production or utilization of insulin and is characterized by hyperglycemia and glycosuria.

"Biologically active" IGF-1 refers to IGF-1 that exhibits a biological property conventionally associated with an IGF-1 agonist or antagonist, such as a property that would allow treatment of one or more of the disorders listed above.

The term "effective amount" refers to an amount of IGF-1 or a peptidomimetic or other compound, including chemical entities, effective to treat a disease or disorder in a mammal. In the case of cancer, for example, the effective amount of the peptide may reduce the number of cancer cells; reduce the tumor size; inhibit (*i.e.*, slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (*i.e.*, slow at least to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; promote apoptosis; and/or relieve to some extent one or more of the symptoms associated with the disorder.

A "precipitant" is an agent in a reservoir solution that precipitates IGF-1 when mixed with an aqueous solution of IGF-1 and allowed to equilibrate so as to form IGF-1 crystals. Examples include chaotropic agents such as ammonium sulfate, polyethylene glycols (of a wide variety of molecular weights ranging, for example, from about 2000 to 20,000), sodium citrate, sodium cacodylate, or a mixture thereof.

A "reservoir solution" is a solution of a precipitant and any other ingredient needed to provide IGF-1 crystals, for example, a detergent such as $C_{12}E_9$ (nonaethylene glycol monododecyl ether, nonaethylene glycol monolauryl ether, polyoxyethylene (9) ether), $C_{12}E_8$ (octaethylene glycol monododecyl ether, octaethylene glycol monolauryl ether, polyoxyethylene (8) lauryl ether), dodecyl-beta-D-maltopyranoside, lauric acid sucrose ester, cyclohexyl-pentyl-beta-D-maltoside, nonaethylene glycol octylphenol ether, cetyltrimethylammonium bromide, decyl-beta-D-maltopyranoside, lauryldimethylamine oxide, cyclohexyl-pentyl-beta-D-maltoside, n-dodecylsulfobetaine, 3-(dodecyldimethylammonio)propane-1-sulfonate, nonyl-beta-D-glucopyranoside, octyl-beta-D-thioglucopyranoside, OSG, N, N-dimethyldecylamine-beta-oxide, methyl-6-O-(N-heptylcarbamoyl)-alpha-D-glycopyranoside, sucrose monocaproylate, heptyl-beta-D-thioglucopyranoside, octyl-beta-D-glucopyranoside, cyclohexyl-propyl-beta-D-maltoside, cyclohexylbutanoyl-N-hydroxyethyleglucamide, n-decylsulfobetaine, 3-(decyldimethylammonio)propane-1-sulfonate, octanoyl-N-methylglucamide, hexyl-beta-D-glucopyranoside, and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. Preferably, the detergent is N, N-bis(3-D-gluconamidopropyl)-deoxycholamine.

"Recrystallization" refers to the procedure, after the initial crystals are grown and determined not to be very large or useful, of adding a substance to the crystals, such as methyl pentanediol, which has the effect of dissolving the crystals, but not diluting anything else much in the crystallization mixture. Then

over the course of several days, as the crystallization droplet re-equilibrates with its reservoir solution, the crystals regrow, but this time much larger and more well ordered.

The term "associating with" refers to a condition of proximity between IGF-1 and a chemical entity, or portions thereof. The association may be non-covalent, wherein the juxtaposition is energetically favored by hydrogen bonding, van der Waals interaction, or electrostatic interaction, or it may be a covalent association.

The term "binding site" refers to any or all of the sites where a chemical entity binds or associates with IGF-1.

The term "structural coordinates" refers to the coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of x-rays by the atoms (scattering centers) of a molecule in crystal form. The diffraction data can be used to calculate an electron density map of the repeating units of the crystal. Those skilled in the art will understand that the data obtained are dependent upon the particular system used, and hence, different coordinates may in fact describe the same crystal if such coordinates define substantially the same relationship as those described herein. An electron density map may be used to establish the positions of the individual atoms within the unit cell of the crystal.

Those of skill in the art understand that a set of structural coordinates determined by x-ray crystallography is not without standard error. Appendix 1 shows the atomic coordinates of IGF-1. For the purpose of this invention, any set of structural coordinates of IGF-1 that have a root mean square deviation of equivalent protein backbone atoms of less than about 2 Å when superimposed--using backbone atoms--on the structural coordinates in Appendix 1 shall be considered identical. Preferably, the deviation is less than about 1 Å and more preferably less than about 0.5 Å.

The term "heavy-atom derivatization" refers to a method of producing a chemically modified form of a crystallized IGF-1. In practice, a crystal is soaked in a solution containing heavy metal atom salts, or organometallic compounds, e.g., lead chloride, gold thiomalate, thimerosal, or uranyl acetate, which can diffuse through the crystal and bind to the surface of the protein. The location of the bound heavy metal atom(s) can be determined by x-ray diffraction analysis of the soaked crystal. This information can be used to generate the phase information used to construct the three-dimensional structure of the molecule.

The term "unit cell" refers to a basic shaped block. The entire volume of a crystal may be constructed by regular assembly of such blocks. Each unit cell comprises a complete representation of the unit of pattern, the repetition of which builds up the crystal.

The term "space group" refers to the arrangement of symmetry elements of a crystal.

The term "molecular replacement" refers to a method that involves generating a preliminary structural model of a crystal whose structural coordinates are unknown, by orienting and positioning a molecule whose structural coordinates are known, e.g., the IGF-1 coordinates in Appendix 1, within the unit cell of the unknown crystal, so as to best account for the observed diffraction pattern of the unknown crystal. Phases can then be calculated from this model, and combined with the observed amplitudes to give an approximated Fourier synthesis of the structure whose coordinates are unknown. This in turn can be subject to any of the several forms of refinement to provide a final accurate structure of the unknown crystal. (See, e.g., Lattman, E., "Use of the Rotation and Translation Functions," Methods in Enzymology, 115: 55-77 (1985); Rossman, ed., "The Molecular Replacement Method," Int. Sci. Rev. Ser. No. 13

(Gordon and Breach: New York, 1972)). Using the structural coordinates of IGF-1 provided by this invention, molecular replacement may be used to determine the structural coordinates of a crystalline co-complex, unknown ligand, mutant, or homolog, or of a different crystalline form of IGF-1. Additionally, the claimed crystal and its coordinates may be used to determine the structural coordinates of a chemical entity that associates with IGF-1.

The term "chemical entity" or "compound" as used herein means any molecule, molecular complex, compound, peptidomimetic, or fragment thereof that is not IGF-1. Preferably it is a molecule with high oral bioavailability, such as an organic chemical molecule, or a peptide.

B. Modes for Carrying out the Invention

The following detailed description of the invention encompasses the crystal structure of IGF-1, methods of making an IGF-1 crystal, and methods of using an IGF-1 crystal and its structural coordinates.

a. Crystal structure of IGF-1

The claimed invention provides crystals of IGF-1 as well as the structure of IGF-1 determined therefrom. Specifically, the claimed invention provides crystals of IGF-1 having approximately the following dimensions: a=31.831 Å, b=71.055 Å, c=65.995 Å, $\alpha=\beta=\gamma=90.000°$. It has a symmetry, or space group, of $C222_1$. The ribbon structure thereof is shown in Figure 2 having three helices, with the N-terminal B-region corresponding to residues 3-28, the C-region from residues 29-34, a stretch of poorly ordered residues from residues 35-40, and the A-region from residues 42-62. The D-region (residues 63-70) is essentially disordered. Figures 4 and 7 show that the detergent used in the crystallization binds into a small hydrophobic cleft at the base of the B-helix of the structure. The IGF-1 can form a dimer in the crystal, as shown in Fig. 5, wherein the two tails are positioned at the dimer interface. The buried surface area is 689 $Å^2$/monomer, which is 1378 $Å^2$ total. The residues important for IGF-1R binding cluster at the dimer interface, as shown in Figure 6.

The characteristics of the claimed IGF-1 crystal are further described in the Example herein and the structural coordinates thereof are provided in Appendix 1.

b. Methods of making an IGF-1 crystal

In various embodiments, the claimed invention relates to methods of preparing crystalline forms of IGF-1 by first providing an aqueous solution comprising IGF-1. A reservoir solution comprising a precipitant is then mixed with a volume of the IGF-1 solution and the resultant mixed volume is then crystallized. In a preferred step the crystals are again dissolved and recrystallized. An example of a reagent that can be used for recrystallization is methyl pentanediol, which is preferred. The crystals are typically dissolved with this reagent in a small amount to minimize dilution effects of the other reagents and left to regrow for a period of time. In an optional step, the crystalline IGF-1 is isolated from the mixed volume. Preferably, the IGF-1 is obtained from a prokaryotic cell, more preferably a bacterial cell, most preferably *E. coli*. Preferably it is secreted into the periplasm and prepared as described in U.S. Pat. No. 5,723,310.

The concentration of IGF-1 in the aqueous solution may vary, but is preferably about 1 to 50 mg/ml, more preferably about 5 to 15 mg/ml. Similarly, precipitants used in the invention may vary, and may be selected from any precipitant known in the art. Preferably, the precipitant is selected from the group consisting of sodium citrate, ammonium sulfate, polyethylene glycol, sodium cacodylate, or a mixture thereof. More preferably the precipitant is polyethylene glycol buffered with sodium citrate or sodium

cacodylate. Any concentration of precipitant may be used in the reservoir solution; however, it is preferred that the concentration be about 20 to 25% if polyethylene glycol, and about 1 to 10 M if sodium citrate, ammonium sulfate, or sodium cacodylate. Preferably, the reservoir solution further comprises a detergent. Preferably, the detergent is present in an amount of about 10 to 50 mM. Also preferably the detergent is N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. The pH of the reservoir solution may also be varied, preferably between about 4 to 10, most preferably about 6.5.

One skilled in the art will understand that each of these parameters can be varied without undue experimentation and acceptable crystals will still be obtained. In practice, once the appropriate precipitating agents, buffers, or other experimental variables are determined for any given growth method, any of these methods or any other methods can be used to grow the claimed crystals. One skilled in the art can determine the variables depending upon one's particular needs.

Various methods of crystallization can be used in the claimed invention, including vapor diffusion, batch, liquid-bridge, or dialysis crystallization. Vapor diffusion crystallization is preferred. See, e.g. McPherson *et al.*, Preparation and Analysis of Protein Crystals, Glick, ed. (John Wiley & Co., 1982), pp. 82-159; Jancarik *et al.*, J. Appl. Crystallogr., 24: 409-411 (1991).

In vapor diffusion crystallization, a small volume (i.e., a few milliliters) of protein solution is mixed with a solution containing a precipitant. This mixed volume is suspended over a well containing a small amount, i.e. about 1 ml, of precipitant. Vapor diffusion from the drop to the well will result in crystal formation in the drop.

The dialysis method of crystallization utilizes a semipermeable size-exclusion membrane that retains the protein but allows small molecules (i.e. buffers and precipitants) to diffuse in and out. In dialysis, rather than concentrating the protein and the precipitant by evaporation, the precipitant is allowed to slowly diffuse through the membrane and reduce the solubility of the protein while keeping the protein concentration fixed.

The batch methods generally involve the slow addition of a precipitant to an aqueous solution of protein until the solution just becomes turbid; at this point the container can be sealed and left undisturbed for a period of time until crystallization occurs.

Thus, applicants intend that the claimed invention encompass any and all methods of crystallization. One skilled in the art can choose any of such methods and vary the parameters such that the chosen method results in the desired crystals.

The most preferred method of crystallization involves the method wherein the IGF-1, after isolation from the cell and formulation in, for example, an acetate, citrate, or succinate buffer, as described, for example, in U.S. Pat. No. 5,681,814 and WO 99/51272, is optionally desalted if necessary to a pH of about 4-5, preferably about 4.5, to form an aqueous solution. Then, a droplet of the aqueous solution is mixed with about 24% polyethylene glycol buffered to about pH 6.5 with either about 0.1M sodium citrate or about 0.1M sodium cacodylate and with about 1 µl of about 1.4 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholamine as detergent. This solution is then equilibrated by vapor diffusion crystallization with about 1 mL of about 24% polyethylene glycol buffered to about pH 6.5 with either about 0.1M sodium citrate or about 0.1M sodium cacodylate until crystallization droplets are formed, usually about 4-5 days.

Then about 2 µl of about 100% methyl pentanediol is added to the crystallization droplets so as to dissolve the crystals overnight and thereby form new crystals, usually within a week's time.

The crystal structure was determined by combined anomalous scattering from intrinsic sulfur and fortuitous bromide ion as discussed in detail in the Example below.

c. Methods of using an IGF-1 crystal and its coordinates

The crystalline IGF-1 herein can be used for various purposes. For example, the crystallization process itself further purifies the IGF-1 to homogeneity. Thus, one such purpose is to provide a highly purified IGF-1 that can be used as a standard or control in a diagnostic setting, for example, as a molecular weight marker, or as an ELISA, radioassay, or radioreceptor assay control. Moreover, crystalline IGF-1 is stable at room temperature, can be lyophilized readily, and is less apt to degrade than less pure compositions.

In another use for the invention herein, crystals of IGF-1 of a size and quality to allow performance of x-ray diffraction studies enable those of skill in the art to conduct studies relating to the binding properties of IGF-1, as well as the binding properties of IGFBPs, IGF-1 receptors, and ALS that associate with the IGF-1.

Furthermore, structural information derived from a peptide crystal structure can be used for the identification of chemical entities, for example, small organic and bioorganic molecules such as peptidomimetics and synthetic organic molecules that bind IGF-1 and preferably block or prevent an IGF-1-mediated or -associated process or event, or that act as IGF-1 agonists. An exemplary approach to such a structure-based compound design is described in Structure Based Drug Design, Pandi Veerapandian, ed. (Marcell Dekker: New York 1997).

By way of example, having determined the three-dimensional structure of the IGF-1, the skilled artisan constructs a model of the IGF-1 such as those depicted in Figures 2 and 5. Since every atom of a peptide or polypeptide can be depicted as a sphere of the appropriate van der Waals radius, a detailed surface map of the folded IGF-1 can be constructed. The surface that results is known as the van der Waals surface. The "solvent-accessible surface" is the surface that is accessible to a chemical probe, a water molecule herein, and is constructed by rolling a water molecule of appropriate radius on the outside of the peptide maintaining contact with the van der Waals surface. Those parts of the van der Waals surface that contact the surface of the water molecule define a continuous surface known as the "solvent-accessible surface." (Creighton, Thomas E., Proteins: structure and molecular properties, 2nd. ed. (W. H. Freeman and Company, 1984), pp227-229).

Such chemical entities presenting a solvent-accessible surface that mimics the solvent-accessible surface of the IGF-1 can be constructed by those skilled in the art. By way of example, the skilled artisan can search three-dimensional structural databases of compounds to identify those compounds that position appropriate functional groups in similar 3-dimensional structural arrangement, then build combinatorial chemistry libraries around such chemical entities to identify those with high affinity.

One approach enabled by this invention is the use of the structural coordinates of IGF-1 to design chemical entities that bind to or associate with IGF-1 and alter the physical properties of the chemical entities in different ways. Thus, properties such as, for example, solubility, affinity, specificity, potency, on/off rates, or other binding characteristics may all be altered and/or maximized.

One may design desired chemical entities by probing an IGF-1 crystal with a library of different entities to determine optimal sites for interaction between candidate chemical entities and IGF-1. For example, high-resolution x-ray diffraction data collected from crystals saturated with solvent allows the determination of where each type of solvent molecule adheres. Small molecules that bind tightly to those sites can then be designed and synthesized and tested for the desired activity. Once the desired activity is obtained, the molecules can be further altered to maximize desirable properties.

The invention also contemplates computational screening of small-molecule databases or designing of chemical entities that can bind in whole or in part to IGF-1. They may also be used to solve the crystal structure of mutants, co-complexes, or the crystalline form of any other molecule homologous to, or capable of associating with, at least a portion of IGF-1.

One method that may be employed for this purpose is molecular replacement. An unknown crystal structure, which may be any unknown structure, such as, for example, another crystal form of IGF-1, an IGF-1 mutant or peptide, or a co-complex with IGF-1, or any other unknown crystal of a chemical entity that associates with IGF-1 that is of interest, may be determined using the structural coordinates as set forth in Appendix I. Co-complexes with IGF-1 may include, but are not limited to, IGF-1-IGFBP-3, IGF-1-IGFBP-3-ALS, IGF-1-receptor, IGF-1-peptide, or IGF-1-small molecule. This method will provide an accurate structural form for the unknown crystal far more quickly and efficiently than attempting to determine such information without the invention herein.

The information obtained can thus be used to obtain maximally effective inhibitors or agonists of IGF-1. The design of chemical entities that inhibit or agonize IGF-1 generally involves consideration of at least two factors. First, the chemical entity must be capable of physically or structurally associating with IGF-1. The association may be any physical, structural, or chemical association, such as, for example, covalent or noncovalent bonding, or van der Waals, hydrophobic, or electrostatic interactions.

Second, the chemical entity must be able to assume a conformation that allows it to associate with IGF-1. Although not all portions of the chemical entity will necessarily participate in the association with IGF-1, those non-participating portions may still influence the overall conformation of the molecule. This in turn may have a significant impact on the desirability of the chemical entity. Such conformational requirements include the overall three-dimensional structure and orientation of the chemical entity in relation to all or a portion of the binding site.

The potential inhibitory or binding effect of a chemical entity on IGF-1 may be analyzed prior to its actual synthesis and testing by the use of computer-modeling techniques. If the theoretical structure of the given chemical entity suggests insufficient interaction and association between it and IGF-1, the need for synthesis and testing of the chemical entity is obviated. However, if computer modeling indicates a strong interaction, the molecule may then be synthesized and tested for its ability to bind to IGF-1. Thus, expensive and time-consuming synthesis of inoperative compounds may be avoided.

An inhibitory or other binding compound of IGF-1 may be computationally evaluated and designed by means of a series of steps in which chemical entities or fragments are screened and selected for their ability to associate with the individual binding sites of IGF-1.

Thus, one skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with IGF-1. This process may begin by visual inspection of, for example, the

binding site on a computer screen based on the IGF-1 coordinates in Appendix 1. Selected fragments or chemical entities may then be positioned in a variety of orientations, or "docked," within an individual binding pocket of IGF-1. Docking may be accomplished using software such as Quanta and Sybyl, followed by energy minimization and molecular dynamics with standard molecular mechanics force fields, such as CHARMM and AMBER.

Specialized computer programs may be of use for selecting interesting fragments or chemical entities. These programs include, for example, GRID, available from Oxford University, Oxford, UK; MCSS or CATALYST, available from Molecular Simulations, Burlington, MA; AUTODOCK, available from Scripps Research Institute, La Jolla, CA; DOCK, available from University of California, San Francisco, CA, and XSITE, available from University College of London, UK.

Once suitable chemical entities or fragments have been selected, they can be assembled into an inhibitor or agonist. Assembly may be by visual inspection of the relationship of the fragments to each other on the three-dimensional image displayed on a computer screen, in relation to the structural coordinates disclosed herein.

Alternatively, one may design the desired chemical entities *de novo*, experimentally, using either an empty binding site or optionally including a portion of a molecule with desired activity. Thus, for example, one may use solid-phase screening techniques where either IGF-1 or a fragment thereof, or candidate chemical entities to be evaluated, are attached to a solid phase, thereby identifying potential binders for further study.

Basically, any molecular modeling techniques may be employed in accordance with the invention; these techniques are known, or readily available to those skilled in the art. It will be understood that the methods and compositions disclosed herein can be used to identify, design, or characterize not only entities that will associate or bind to IGF-1, but alternatively to identify, design, or characterize entities that, like IGF-1, will bind to the receptor, thereby disrupting the IGF-1-receptor interaction. The claimed invention is intended to encompass these methods and compositions broadly.

Once a compound has been designed or selected by the above methods, the efficiency with which that compound may bind to IGF-1 may be tested and modified for the maximum desired characteristic(s) using computational or experimental evaluation. Various parameters can be maximized depending on the desired result. These include, but are not limited to, specificity, affinity, on/off rates, hydrophobicity, solubility, and other characteristics readily identifiable by the skilled artisan.

Additionally, the invention is useful for the production of small-molecule drug candidates. Thus, the claimed crystal structures may be also used to obtain information about the crystal structures of complexes of the IGF-1 and small-molecule inhibitors. For example, if the small-molecule inhibitor is co-crystallized with IGF-1, then the crystal structure of the complex can be solved by molecular replacement using the known coordinates of IGF-1 for the calculation of phases. Such information is useful, for example, for determining the nature of the interaction between the IGF-1 and the small-molecule inhibitor, and thus may suggest modifications that would improve binding characteristics such as affinity, specificity, and kinetics.

d. Other methods

The invention herein is also useful in providing a method of identifying indirect agonists of IGF-1 based on the inhibitory properties of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine with respect to IGFBPs. This method comprises the steps of: comparing the ability of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine to inhibit binding of IGFBP-1 or -3 to IGF-1 with the ability of a candidate IGF-1 indirect agonist to inhibit such binding; and determining whether the candidate IGF-1 indirect agonist can inhibit such binding at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholamine can so inhibit the binding.

Preferably the comparison is accomplished by competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and the candidate IGF-1 indirect agonist, using IC_{50} to measure ability to inhibit IGFBP binding. In a more preferred embodiment, inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholamine or the candidate agonist molecule with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based assay, such as an ELISA.

The invention further provides a method of identifying indirect agonists of IGF-1 comprising co-crystallizing the candidate agonist with IGF-1 to form a co-crystalline structure and determining if the candidate agonist molecule binds to one or both of two patches on IGF-1. The first patch contains the amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and the second patch contains the amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55. For purposes herein, binding means that there is at least one contact between each listed amino acid residue of a given patch and the candidate agonist molecule that is less than or equal to 6 angstroms in the co-crystalline structure. Such a candidate agonist molecule will have the property of inhibiting binding of IGFBP-1 or IGFBP-3 to IGF-1. The preferred such candidate agonist molecule will inhibit binding of IGFBP-1 or -3 to IGF-1 at least as well as N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. More preferred is the method wherein inhibition of binding is measured using a competition assay between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and the candidate agonist molecule. Most preferred is the method wherein inhibition of binding is measured by pre-incubating N, N-bis(3-D-gluconamidopropyl)-deoxycholamine or the candidate agonist molecule with IGF-1 expressed on bacteriophage particles and measuring residual binding of IGF-1 to IGFBP-1 or IGFBP-3 in a plate-based ELISA assay.

The N, N-bis(3-D-gluconamidopropyl)-deoxycholamine detergent herein can be used as a template to perform design of small-molecule drugs that elicit the same effect as the detergent (compete with IGF-1 for IGFBP binding and subsequent disruption of the interaction of IGFBP with IGF-1 to free IGF-1 *in situ* so that it is active and will interact with the receptor. As opposed to the other detergents tested in the Examples below, N, N-bis(3-D-gluconamidopropyl)-deoxycholamine lacks an oxygen atom at position C10. This region of the detergent is in close contact with the side-chain atoms of residues Leu 5, Leu 54, and Leu 57 of IGF-1. Molecules with this same type of conformation would work as indirect IGF-1 agonists.

The indirect agonist so identified can be used in a method for treating an agonist disorder wherein an effective amount of the indirect agonist of IGF-1 is administered to a mammal with such a disorder. Hence, such agonist may be used therapeutically in a pharmaceutical preparation, for example, in clinical

trials or commercialized for the agonist disorders as defined herein. Thus, the formulation of the indirect agonist herein can be used to treat any condition that would benefit from treatment with IGF-1, including, for example, diabetes, chronic and acute renal disorders, such as chronic renal insufficiency, necrosis, *etc.*, obesity, hyperinsulinemia, GH-insufficiency, Turner's syndrome, short stature, undesirable symptoms associated with aging such as increasing lean-mass-to-fat ratios, immuno-deficiencies including increasing CD4 counts and increasing immune tolerance, catabolic states associated with wasting, *etc.*, Laron dwarfism, insulin resistance, and so forth.

For therapeutic use, the indirect agonist composition herein may be directly administered to the mammal by any suitable technique, including orally, parenterally, intranasally, or intrapulmonarily, and can be administered locally or systemically. The specific route of administration will depend, e.g., on the medical history of the patient, including any perceived or anticipated side or reduced effects using IGF-1, and the disorder to be treated. Examples of parenteral administration include subcutaneous, intramuscular, intravenous, intraarterial, and intraperitoneal administration. Most preferably, the administration is by continuous infusion (using, e.g., minipumps such as osmotic pumps), or by injection (using, e.g., intravenous or subcutaneous means). The administration may also be as a single bolus or by slow-release depot formulation. Most preferably, the direct agonist is administered orally or by infusion or injection, at a frequency of, preferably, one-half, once, twice, or three times daily, most preferably daily.

The agonist composition to be used in the therapy will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical condition of the individual patient (especially the side effects of treatment with the agonist), the site of delivery of the agonist composition, the method of administration, the scheduling of administration, and other factors known to clinical practitioners. The "effective amount" of agonist for purposes herein is thus determined by such considerations and must be an amount that treats the disorder in question.

As a general proposition, the total pharmaceutically effective amount of agonist administered parenterally per dose will be in the range of about 1 μg/kg/day up to about 100 mg/kg/day, preferably 10 μg/kg/day up to about 10 mg/kg/day. If given continuously, the agonist is generally administered in doses of about 1 μg/kg/hour up to about 100 μg/kg/hour, either by about 1-4 injections per day or by continuous subcutaneous infusions, for example, using a minipump or a portable infusion pump. An intravenous bag solution may also be employed. The key factor in selecting an appropriate dose is the result obtained as measured by criteria as are deemed appropriate by the practitioner. If the agonist is administered together with insulin, the latter is used in lower amounts than if used alone, down to amounts which by themselves have little effect on blood glucose, i.e., in amounts of between about 0.1 IU/kg/24 hour to about 0.5 IU/kg/24 hour.

For parenteral administration, in one embodiment, the agonist is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, i.e., one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

Generally, the formulation is prepared by contacting the agonist uniformly and intimately with a liquid carrier or a finely divided solid carrier or both. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low-molecular-weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; glycine; amino acids such as glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; nonionic surfactants such as polysorbates, poloxamers, or PEG; and/or neutral salts, e.g., NaCl, KCl, $MgCl_2$, $CaCl_2$, etc.

The agonist is typically formulated individually in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 4.5 to 8. The final formulation, if a liquid, is preferably stored at a temperature of about 2-8°C for up to about four weeks. Alternatively, the formulation can be lyophilized and provided as a powder for reconstitution with water for injection that is stored as described for the liquid formulation.

The agonist to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic agonist compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The agonist ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. The disclosures of all literature and patent citations mentioned herein are expressly incorporated by reference.

EXAMPLE 1

Crystallization and Characterization of IGF-1 Crystals

Crystallization of IGF-1 and data collection

Recombinant human IGF-1 (rhIGF-1) was obtained as described in the Examples of U.S. Pat. No. 5,723,310 using a polymer/salt combination for phase-forming species and formulated as described in the Examples of U.S. Pat. No. 5,681,814 (acetate, NaCl, polysorbate 20, and benzyl alcohol). Specifically, the initial isolation of IGF-1 from *E. coli* was achieved using aqueous two-phase separation (Hart *et al.*, Bio/Technology, 12: 1113-1117 (1994)), followed by refolding (Hart *et al.*, Biotechnol. Appl. Biochem., 20: 217-232 (1994)), and subsequent chromatographic purification, including large-scale reverse-phase high-performance liquid chromatography (Olson *et al.*, J. Chromatogr., A675: 101-112 (1994)). It was placed in

a vial containing 7 ml of 10 mg/ml rhIGF-1. Prior to crystallization, the IGF-1 was desalted into 0.15 M NaCl and 20 mM sodium acetate (pH 4.5), and diluted to a final concentration of 10 mg/ml. Initially, crystallization trials were set up in the presence of 1 mM of an IGF-1-binding peptide. However, no peptide was ever observed in the crystal, and crystals grown in the absence of the peptide were later shown to be isomorphous to the specimen reported here. A 4-µl droplet of the IGF-1 solution was mixed with 5 µl of reservoir solution (24% polyethylene glycol 3350 buffered to pH 6.5 with 0.1M sodium cacodylate) and 1 µl of 14 mM of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine, which is obtained in a CRYSTAL SCREEN™ reagent kit used for crystallization condition screenings and available from Hampton Research, Inc., Laguna Nigel, CA. This solution was allowed to equilibrate via vapor diffusion (Jancarik *et al., supra*) with 1 ml of reservoir solution. Thus, a drop of the mixture was suspended under a plastic cover slip over the reservoir solution. Small crystals with a thin, plate-like morphology appeared within 4-5 days. At this point, 2 µl of 100% methyl pentanediol (MPD) (to a final concentration of 20%) was added to the crystallization droplet, and the crystals dissolved overnight. Within 1 week, crystals reappeared and grew to final dimensions of 0.2 mm X 0.1mm X 0.05 mm with noticeably sharper edges. These crystals were used for all subsequent analysis.

Those of skill in the art will appreciate that the aforesaid crystallization conditions can be varied. By varying the crystallization conditions, other crystal forms of IGF-1 may be obtained. Such variations may be used alone or in combination, and include, for example, varying final protein concentrations between about 5 and 35 mg/ml; varying the IGF-1-to-precipitant ratio, varying precipitant concentrations between about 20 and 30% for polyethylene glycol, varying pH ranges between about 5.5 and 7.5, varying the concentration or type of detergent, varying the temperature between about -5 and 30°C, and crystallizing IGF-1 by batch, liquid bridge, or dialysis methods using the above conditions or variations thereof. See McPherson *et al.* (1982), *supra.*

Characterization of IGF-1 crystals

A single crystal was transferred from the mother liquor to a cryo-protectant solution consisting of 25% (w/v) polyethylene glycol 3350, 30% MPD, 0.2 M sodium cacodylate pH 6.5, 2.8 mM of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine, and 1 M NaBr. The diffraction was to 1.8 Å. After 30 seconds in this solution, the crystal was flash-cooled by plunging it into liquid nitrogen. The technique of freezing the crystals essentially immortalizes them and produces a much higher quality data set. All subsequent manipulations and x-ray data collection were performed at 100° Kelvin.

A 4-wavelength MAD data set was collected at beamline 9-2 at the Stanford Synchrotron Radiation Laboratory, with the order of the data sets as follows: Br peak ($\lambda 1$), low-energy remote ($\lambda 2$), Br inflection ($\lambda 3$), and high-energy remote ($\lambda 4$). The Br peak and inflection points were estimated from fluorescence scans of the crystal, and the low-energy remote was chosen to be 1.54 angstroms, to maximize the small sulfur anomalous signal at this wavelength while minimizing absorption effects. No inverse beam geometry was used. Data reduction was performed using Denzo and Scalepack (Otwinowski and Minor, Methods in Enzymology, 276: 307-326 (1997)). To determine the most accurate scale and *B*-factors possible, data for all four wavelengths were initially scaled together, assuming no anomalous signal. The scale and *B*-factors determined from this scaling run were then applied to each of the four data sets.

The crystals belong to space group $C222_1$ with unit cell dimensions or constants of: a=31.83 Å, b=71.06 Å, and c=66.00 Å. $\alpha=\beta=\gamma=90.000°$. The asymmetric unit of the crystals contained a monomer of IGF-1 bound to a single detergent molecule, yielding a Matthew's coefficient of 2.4 $Å^3$/Da, or 48.1% solvent. The solvent content of the crystals was about 55%.

Structure determination

Initial attempts at determining the structure of IGF-1 by molecular replacement, using either the available NMR models of IGF-1 or the crystal structure of insulin, were unsuccessful. For this reason, the structure was determined *de novo* by Br multiwavelength anomalous dispersion (MAD) (Dauter *et al.,* Acta Crystallogr. D56: 232-237 (2000)).

The coordinates of the single-bound bromide were determined by manual inspection of the anomalous and dispersive difference Patterson maps. The hand ambiguity was resolved by phase refinement using the program SHARP (De La Fortelle and Bricogne, Methods in Enzymology, 276: 472-494 (1997)) from Global Phasing Limited, 43 Newton Road, Cambridge CB2 2AL, ENGLAND, followed by examination of anomalous-difference Fourier maps calculated using the λ2 Bijvoet differences. A cluster of six peaks for one hand of the Br coordinates was consistent with the disulfide structure of insulin (PDB entry: 1ZNI). These six peaks correspond to the six Cys Sγ atoms in IGF-1; a seventh sulfur (Met 59 Sδ) was never detected in anomalous-difference Fourier maps, presumably due to its higher temperature factor (36.7 $Å^2$). At this point, the six Cys Sγ positions were included in the phase refinement, with the λ1 data set used as a reference. Throughout the phase refinement, the Br f" was refined for the λ1 data set, f' and f" were refined for λ3, and both were kept fixed for data sets λ2 and λ4; the f" and f' values for sulfur were kept fixed at the theoretical values for each wavelength. The small anomalous signal from the sulfur atoms had a modest effect on the phasing statistics, but the resulting electron-density maps showed improved connectivity, especially in the less well ordered regions of IGF-1.

Density modification (solvent flattening and histogram mapping) was performed using DM (Collaborative Computational Project Number 4, Acta Crystallogr., D50: 760-763 (1994); Cowtan, Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography, 31: 34-38 (1994)), and the resulting electron-density maps were of high quality. Approximately 50% of the structure, corresponding to the three helical regions of IGF-1, was built directly into the experimental electron-density maps using the programs O (Jones *et al.,* Acta Crystallogr., A47: 110-119 (1991)) and QUANTA (version 97.0, MSI, San Diego, CA). Several rounds of phase combination using Sigmaa (Collaborative Computational Project Number 4, *supra*; Read, Acta Crystallogr., A42: 140-149 (1986)) allowed the remainder of the molecule to be modeled. Atomic positional and restrained *B*-factor refinement utilized the maximum-likelihood target function of CNX (Brünger *et al.,* Acta Crystallogr., D54: 905-921 (1998) and MSI, San Diego, CA), coupled with a "mask"-type bulk solvent correction and anisotropic overall *B*-factor scaling.

The final model contains residues 3-34 and 41-64 of IGF-1, one N, N-bis(3-D-gluconamidopropyl)-deoxycholamine molecule, 1 Br^-, and 50 water molecules. The model was refined against the λ3 data set, since the data statistics demonstrated this data set to be of higher quality than the others. All data from 20- to 1.8-angstrom resolution were included in the refinement, with no application of a sigma cutoff. Secondary structure assignments were made with the program PROMOTIF (Jones *et al., supra;* Hutchinson and Thornton, Protein Science, 5: 212-220 (1996)).

While the well-ordered positions of IGF-1 were essentially identical using the two sets of phases, the more flexible regions of the molecule showed dramatically improved connectivity upon inclusion of the sulfurs in the phasing. Experimental electron density maps showing the turn region of IGF-1 immediately following the first helix (residues 19, 20, and 21) indicate that using the combined Br and S phases resulted in a much more well-connected map than using just the Br phases alone. At this point, using the Br + S phases, about 50% of the molecule could be traced directly into the experimental maps.

Description of the Structure

After several cycles of model building and phase combination, the final model, shown in Figure 2, contains residues 3-34 and 41-64 of IGF-1, a single-bound detergent molecule, and 46 water molecules. The R factor to 1.8 Å is 23.7%, and the free R factor is 26.9%, with good stereochemistry. The N-terminal B-region corresponds to residues 3-28, the C-region from 29-34, a stretch of poorly ordered residues from 35-40, and the A-region from 42-62. The D-region (63-70) is essentially disordered.

The structure of IGF-1 is similar to insulin (see Figure 3), with a Root-Mean-Squared-Deviation (RMSD) of 3Å over backbone atoms that are conserved between the two molecules. Most of these deviations occur in the flexible regions, and when only the helical regions are considered, the RMSD between alpha-carbon atoms is about 0.47Å. The major difference is the extension of the C-region, for which there is no counterpart in mature insulin, away from the body of the molecule. This loop contains many of the residues that are known to be important for receptor binding.

An extensive alanine-scan mutagenesis study on IGF-1 has shown which residues are important for binding to IGFBP-1 and IGFBP-3 (Dubaquie and Lowman, *supra*). The residues that bind to IGFBP-3 are similar to those that bind IGFBP-1, although IGFBP-3 is believed to depend more on backbone interactions and is less severely affected by alanine mutations. There is no one dramatic spot where residues important for IGFBP-1 and IGFBP-3 binding are clustered, and mutations that impair binding are scattered all over the molecule. There appears to be a slight clustering of sites at the N-terminus, with many of these sites being intrinsically hydrophobic.

As shown in Figures 4 and 7, the detergent molecule binds into a small hydrophobic cleft at the base of the B-helix. There are several direct side-chain contacts to the detergent from residues 5, 7, and 10. Despite the overlap of the detergent binding site with a portion of the IGFBP-1/IGFBP-3 binding epitope, the preliminary results suggest, without being limited to any one theory, that the detergent does not inhibit binding of these proteins to IGF-1. The opposite face of the detergent is making a symmetry contact to the opposite face of IGF-1.

As shown in Figure 5, there is only one large crystal packing contact between symmetry-related IGF-1 molecules, which results in a symmetric homodimer. The buried surface area is 1378 $Å^2$, which is in the range of physiologically relevant protein-protein interfaces.

Figure 6 shows that the residues known to be important for receptor binding cluster at this dimer interface. Shown are Tyr24, Thr29, Tyr31, and Tyr60. Mutation of these residues results in anywhere from 6-20X loss in affinity for receptor for individual mutations, or 240->1200X loss in affinity for double mutations. Also shown are Phe23 and Phe25, which are interchangeable with Phe24 and Tyr26 of insulin, with no loss of affinity.

Further Description of the Structure

IGF-1 is composed primarily of three helical segments corresponding to the B-helix (IGF-1 residues 7-18) and two A-helices (IGF-1 residues 43-47 and 54-58) of insulin. The hydrophobic core is essentially identical to that described for the NMR structures of IGF-1, including the three disulfide linkages between Cys 6 and Cys 48, Cys 18 and Cys 61, and Cys 47 and Cys 52, as noted in the references above. Residues 3 through 6 do not form any regular secondary structure, and hence, the structure described herein can be classified as being most similar to the T-form of insulin (Derewenda *et al.*, Nature, 338: 594-596 (1989)). Indeed, when IGF-1 and the T-form of insulin are superimposed on the Cα positions of their respective helical segments (IGF-1 residues 8-19, 42-49, and 54-61; insulin residues B9-B20, A1-A8, and A13-A20) the RMSD is only 0.47 angstroms. As in insulin, residues 18-21 at the end of the B-helix form a type II' β-turn, which redirects the backbone from the B-helix into an extended region. Residues 24-27 form a type VIII ß-turn to accommodate the C-region, which extends away from the core of IGF-1, and interacts with a symmetry-related molecule. Residues 30-33 form a well-defined type II beta-turn, prominently displaying Tyr 31 at the i+1 position. Residues 35-40 have not been modeled, as the electron density in this region is weak and disconnected. Only the first two residues of the D-region (residues 63 and 64) are ordered in the structure.

The C-region of IGF-1 mediates a two-fold symmetric crystal-packing interaction across the *a*-axis of the unit cell. This interaction buries 689 $Å^2$ of solvent-accessible surface area from each molecule of IGF-1, or 1378 $Å^2$ total, and is the largest interface in the crystal. A total of 28 intermolecular contacts of distance 3.6 Å or less are formed via this interface, with the next most extensive crystal packing interaction forming only nine contacts. The core of the interface is dominated by Tyr24 and Pro28 from each monomer, which bury 39 $Å^2$ and 57 $Å^2$ of solvent-accessible surface area, respectively. The aromatic ring of Tyr 31, which lies at the tip of the loop at the furthest point from the core of IGF-1, packs against the phenolic rings of Phe 23 and Phe 25 of the symmetry-related molecule. In addition to these hydrophobic interactions, two main-chain hydrogen bonds (Tyr 31 N-Phe 23 O and Ser 34 N-Asp 20 O) are present in the dimer interface. Residues from the D-region (62-64) are also partially sequestered by this dimer formation. Because of these interactions, most of the C-region in the crystal is well-ordered, providing the first high-resolution view of the conformation of this biologically important loop.

Although 72 detergent compounds, including the similar 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate (CHAPS) and 3-((3-cholamidopropyl)dimethylammonio)-2-hydroxypropanesulfonic acid (CHAPSO) detergents, were screened in crystallization trials, only N, N-bis(3-D-gluconamidopropyl)-deoxycholamine yielded crystals. A single molecule of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine interacts with residues, forming a small hydrophobic cleft on one surface of IGF-1 (Leu 5, Phe 16, Val 17, Leu 54, and Leu 57) (Fig. 7A). The preference for N, N-bis(3-D-gluconamidopropyl)-deoxycholamine is explained, without being limited to any one theory, by the absence of an oxygen atom at position C10 in the detergent molecule. This region of the detergent is in close contact with the side chain atoms of residues Leu 5, Leu 54, and Leu 57 in IGF-1. The opposite face of the detergent mediates a symmetry contact with residues Val 11, Leu 14, and Gln 15 of a symmetry-related IGF-1 molecule. Intriguingly, this face of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine also contacts the edge of the dimer interface, with close contacts to Phe 23 and Phe 25 of the same IGF-1 molecule, as

well as Tyr 31 and Gly 32 of the dimeric partner (Fig. 7B). A more detailed analysis indicates that the detergent binds to two patches of binding pockets of IGF-1. One patch has the amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and the second patch has the amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55. Binding is defined by having at least one contact between each listed amino acid residue and the candidate agonist molecule that is less than or equal to 6 angstroms.

Discussion

The C-region in the IGF-1 crystal structure extends out from the core of the molecule, with residues 30-33 forming a canonical type II beta-turn, and the remainder of the C-region forming a crystallographic dimer with a symmetry-related molecule. Tyr 31 has been implicated as being a critical determinant for IGF-1R binding (Bayne *et al.* (Vol. 264), *supra*; Bayne *et al.* (Vol. 265), *supra*; Cascieri et al., supra), and its location at the tip of this extension places it in an ideal location to interact with a receptor molecule. While this region of IGF-1 is not well-defined by NMR data, the conformation of the C-region in the crystal is likely to reflect a prevalent solution conformation. There is evidence of a reverse turn at the tip of the loop and a hinge bending at the loop termini of IGF-2 (Torres *et al., supra*). Thus, while crystal packing forces undoubtedly help stabilize the orientation of this loop, its conformation appears to be consistent with the solution structure of the closely related IGF-2.

The size of the interface formed by the crystallographic dimer is well within the range of buried surface area in known biological complexes (Janin and Chothia, J. Biol. Chem., 264: 16027-16030 (1990)). In addition, this interaction partially excludes from solvent several of the residues known to be important for binding to the IGF-1R, including Phe 23 (69% buried), Tyr 24 (64% buried), Phe 25 (29% buried), and Tyr 31 (38% buried). Other groups have also reported homodimeric interactions of IGF-1 and IGF-2. Laajoki *et al.*, (2000*), supra*, report that at a concentration of 1 mM, an engineered form of IGF-1 (Long-[Arg^3]IGF-1) partitions into about 20% dimer/80% monomer, a ratio that is in good agreement with the estimate of 3.6 mM K_d. In their NMR study of IGF-2, Torres *et al., supra,* reported that the amide protons of residues in the C-region were slowly exchanging with solvent, suggesting that IGF-2 forms a homodimer in solution. However, despite the significant amount of surface area that is buried upon dimer formation in the crystal, the affinity of IGF-1 for itself is very weak. In addition, the known binding stoichiometry of one IGF-1 molecule per receptor dimer (De Meyts, *supra*) makes it difficult to rationalize the biological significance of IGF-1 dimerization. In conclusion, the IGF-1 dimer in this crystal form results from the high concentration of IGF-1 in the crystallization experiment, and does not represent a physiologically relevant form of the molecule.

The very low quality of NMR spectroscopic data obtained for IGF-1 at near-neutral pH has been attributed to a combination of self-association and internal mobility that leads to a large variation in resonance line width (Cooke *et al., supra*). As a result, NOESY spectra acquired on IGF-1 contain many broad, overlapped peaks and few sharp well-resolved correlations. NOESY spectra collected for IGF-1 in the presence of an excess of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine have a similar appearance. Thus, detergent binding is not sufficient to eliminate the aggregation or inherent flexibility of IGF-1 and does not facilitate characterization of the solution conformation of the protein. Likewise, detergent binding does not alter the aggregation state of IGF-1, as assessed by analytical ultracentrifugation experiments in the

presence of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. This is in contrast to observations in the crystalline state where addition of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine leads to a well-packed crystallographic dimer and crystals that diffract to high resolution. Jansson *et al.*, J. Biol. Chem., 273: 24701-24707 (1998) noted that the lack of NMR assignments in the region immediately surrounding Cys 6, which includes Leu 5 and Gly 7, was indicative of the Cys 6-Cys 48 disulfide undergoing intermediate exchange between a cis and trans configuration. The fact that the detergent binds to one face of the B-helix immediately opposite this disulfide suggests, without being limited to any one theory, that it may serve to stabilize this region of the molecule by more complete packing of the hydrophobic cleft. Indeed, in the crystal structure herein, the Cys 6-Cys 48 is clearly in the trans conformation, and there is no evidence of multiple conformations.

Conclusion

The crystal structure of IGF-1 has been determined using anomalous scattering from the intrinsic sulfur atoms and a Br- ion bound at a fortuitous halide-binding site. The structure is very similar to insulin, with the only major difference being the C-region, which protrudes from the body of the protein and mediates a homodimeric interaction. The amount of buried surface area is consistent with the fact that at neutral pH, IGF-1 undergoes self-association in a concentration-dependent manner. In addition, several residues that are important for receptor binding are found at this dimer interface, suggesting, without being limited to any one theory, that effects on receptor binding by mutation of these residues may be a result of disruption of the dimer, rather than direct contact with the receptor surface.

EXAMPLE 2

Diffusion-based measurement of detergent binding

NMR-derived diffusion measurements were used to estimate the K_d for the interaction between IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine. Samples were prepared in 50 mM phosphate buffer in D_20, pH 6.5 (uncorrected meter reading), and contained: 1.0 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholamine + 0.5 mM IGF-1; 0.5 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholamine + 0.25 mM IGF-1; 0.25 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholamine + 0.125 mM IGF-1; or N, N-bis(3-D-gluconamidopropyl)-deoxycholamine only (1.0, 0.5, or 0.25 mM). All spectra were acquired at 40°C on a Bruker AVANCE 500™ spectrometer (Bruker Analytik GmbH) equipped with a 5-mm triple-axis gradient, triple-resonance probe. Diffusion measurements were made with a bipolar pulse pair method with $\delta = 5$ ms, $\tau = 2$ ms, and $\Delta = 25$ or 40 ms for N, N-bis(3-D-gluconamidopropyl)-deoxycholamine alone or N, N-bis(3-D-gluconamidopropyl)-deoxycholamine + IGF-1, respectively (Wu *et al.*, J. Magn. Reson., Ser. A 115: 260-264 (1995)). Spectra were collected with 128 to 1024 transients as the z-gradient strength was increased from 0.009 to 0.45 $T \bullet m^{-1}$ in 18 equal increments; measurements were made at least twice on each sample. Spectra were processed and peak heights extracted with the program FELIX (v98.0, MSI, San Diego). Diffusion constants, proportion of bound detergent, and resulting K_d were extracted as described by Fejzo *et al.*, Chemistry & Biology, 6: 755-769 (1999). Spectra were also collected on samples containing 1.0 mM 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate, a zwitterionic detergent used for membrane solubilization, and 1.0 mM 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate + 0.5 mM IGF-1. Two-dimensional NOESY spectra (Jeener *et*

al., J. Chem. Phys., 71: 4546-4553 (1979)) were collected on a 0.5-mM sample of IGF-1 in the presence or absence of 1.0 mM N, N-bis(3-D-gluconamidopropyl)-deoxycholamine with a mixing time of 100 ms.

IGF-1 phage ELISA

E. coli cells (XL1-Blue, Stratagene) freshly transformed with the phage vector pIGF-g3 displaying human IGF-1 as described in Dubaquie and Lowman, *supra,* were grown overnight in 5 ml of 2YT medium (Sambrook *et al.*, Molecular Cloning: A Laboratory Handbook (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)). The phage particles displaying IGF-1 were titered against IGFBP-1 and IGFBP-3 to obtain a 500-1000-fold dilution for preincubation with serial dilutions of the detergents and binding protein standards for 45 minutes. Microwell clear polystyrene immunoplates with a MAXISORP™ surface (Nunc, Denmark) were coated with IGFBP-1 or IGFBP-3 protein overnight at 4°C (50 µl at 3 µg/mL in 50 mM carbonate buffer, pH 9.6), blocked with 0.5% TWEEN® 20 polyoxyethylene sorbitan monolaurate (Atlas Chemical Co.), and PBS and washed eight times with PBS and 0.05% TWEEN® 20 polyoxyethylene sorbitan monolaurate. The samples were added to the plates for 30 minutes. Plates were washed eight times with PBS and 0.05% TWEEN® 20 polyoxyethylene sorbitan monolaurate, incubated with 50 µL of 1:10,000 horseradish peroxidase/anti-M13 antibody conjugate (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS and 0.5% BSA for 30 minutes, and then washed eight times with PBS and 0.05% TWEEN® 20 polyoxyethylene sorbitan monolaurate and two times with PBS. Plates were developed using a tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD), stopped with 1.0 H_3P0_4, and read spectrophotometically at 450 nm.

Sedimentation equilibrium analysis

The self-association of IGF-1 was determined by sedimentation equilibrium analysis. The experiments were conducted at 20°C in an OPTIMA™ XL-A/XL-I analytical ultracentrifuge (Beckman Coulter, Inc.). The samples were prepared in 0.1 M citrate buffer, pH 6.5, 75 mM NaCl with a loading concentration from 1 mM to 0.01 mM. The concentration gradients were measured at rotor speeds of 25000 and 30000 rpm at 280 nm or 285 nm using a scanning absorption optical system. The attainment of an equilibrium state was verified by comparing successive scans after approximately 16 hours. The partial specific volume of IGF-1 was calculated from its amino acid composition. The data were fit as a single ideal species or the ideal dimer self-association models using a non-linear least-squares fitting program, NONLIN (Johnson *et al.*, Biophys. J., 36: 578-588 (1981)). The association constants were determined from the best-fit values of the model, returned by non-linear least-squares regression.

Results:

N, N-bis(3-D-gluconamidopropyl)-deoxycholamine binds to IGF-1 in solution.

The affinity of IGF-1 for 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine was ascertained using solution-NMR methods. The chemical shift changes observed during a titration of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine into a 0.5 mM IGF-1 solution suggested that the affinity was submillimolar and not easily measurable from such data. Instead, diffusion measurements were made on samples at varying IGF-1 concentrations containing 2 molar equivalents of detergent and also on several samples of detergent alone (the detergent concentration was always less than the critical micelle concentration of 1.4 mM for N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and 14 mM for 3-((3-cholamidopropyl) dimethylammonio)-1-propane

sulphonate). The decrease in diffusion constant of the detergent in the presence of the protein can be used to estimate the proportion of detergent bound to the protein (Fejzo *et al.*, *supra*). Since the total concentration of detergent and protein is known, a value of the dissociation constant can be determined. At the three protein concentrations studied (0.5 mM, 0.25 mM, and 0.125 mM), K_d values of 220, 440, and 430 μM were obtained, respectively. This technique has routinely been applied to small molecules (several hundred Daltons molecular weight or less) binding to large proteins. In this particular case, the ligand is relatively large (862 Da) and the protein is relatively small (7648 Da); hence, the differential decrease in diffusion constant on binding is small. This increases the uncertainty with which the dissociation constant can be measured. Given this, the data described above suggest that the K_d for the interaction between N, N-bis(3-D-gluconamidopropyl)-deoxycholamine and IGF-1 is 300±150 μM. A similar analysis of the (3-(3-cholamidopropyl) dimethylammonio)-1-propane sulphonate diffusion data suggests that that K_d in this case is greater than 3 mM.

N, N-bis(3-D-gluconamidopropyl)-deoxycholamine blocks IGFBP-1 and IGFBP-3 binding.

To examine the binding epitope of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine on IGF-1, the detergent was preincubated with IGF-1 expressed on bacteriophage particles, and the level of residual binding to IGFBP-1 and IGFBP-3 was measured in a plate-based assay (ELISA). As a control, soluble IGFBP-1 was also tested. As shown in Figure 8, N, N-bis(3-D-gluconamidopropyl)-deoxycholamine inhibited IGF-1 on phage from binding to IGFBP-1 and IGFBP-3 with IC_{50} values of 480±170 μM and 275±152 μM, respectively. These numbers must be interpreted conservatively, however, since the critical micelle concentration of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine (1.4 mM) presents an upper limit on the curve in Figure 8. In contrast to the effect of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine, the closely related detergent 3-((3-cholamidopropyl) dimethylammonio)-1-propane sulphonate did not show any inhibition of binding at any of the concentrations tested up to 1 mM. Despite the limitations of the experiment, the IC_{50} values obtained for N, N-bis(3-D-gluconamidopropyl)-deoxycholamine are in good agreement with the NMR-based estimate of a K_d of ~300 μM for the N, N-bis(3-D-gluconamidopropyl)-deoxycholamine-IGF-1 interaction.

Self-association of IGF-1.

The sedimentation equilibrium data show that IGF-1 undergoes self-association in solution. The average molecular weight increased with increasing protein concentration from 0.01 mM to 1 mM. The average molecular weight at the highest concentration studied (1 mM) is about 37% higher than the monomer molecular weight (10.4 KDa at 1 mM versus 7.6 KDa monomer molecular weight). At concentrations below 0.05 mM, no self-association was observed, and IGF-1 exists only as a monomer in solution at neutral pH. If it is assumed that the higher-molecular-weight species are IGF-1 dimers, the sedimentation data can be fit as a monomer-dimer model with a K_d of 3.6±1.0 mM (Figure 9).

Discussion

Several studies have identified residues in IGF-1 that are important for IGFBP binding (Clemmons *et al.*, Endocrinology, 131: 890-895 (1992); Dubaquie and Lowman, *supra*; Jansson *et al.*, *supra*; Oh *et al.*, (1993), *supra*; Lowman *et al.*, (1998), *supra*; and Dubaquie *et al.*, Endocrinology, 142: 165-173 (2001)). Dubaquie and Lowman, *supra,* identified two distinct patches on IGF-1 that interact with IGFBP-1 and IGFBP-3. Patch I consists of Glu 7, Leu 10, Val 11, Leu 14, Phe 25, Ile 43, and Val 44, while patch 2

consists of Glu 3, Thr 4, Leu 5, Phe 16, Val 17, and Leu 54. In the crystal structure of IGF-1, these two patches are involved in detergent-mediated crystal packing contacts. (Specifically, Patch 1 of the crystal structure of IGF-1 consists of amino acid residues Glu 3, Thr 4, Leu 5, Asp 12, Ala 13, Phe 16, Val 17, Cys 47, Ser 51, Cys 52, Asp 53, Leu 54, and Leu 57, and Patch 2 of the crystal structure of IGF-1 consists of amino acid residues Val 11, Gln 15, Phe 23, Phe 25, Asn 26, Val 44, Phe 49, and Arg 55, wherein binding occurs if there is at least one contact between each listed amino acid residue and the candidate agonist molecule that is less than or equal to 6 angstroms.)

The overlap of the detergent binding site with the IGFBP interaction surfaces is entirely consistent with the observation herein that N, N-bis(3-D-gluconamidopropyl)-deoxycholamine blocks IGFBP-1 and IGFBP-3 binding. In contrast, N, N-bis(3-D-gluconamidopropyl)-deoxycholamine does not inhibit IGF-1R-mediated signaling in a cell-based receptor activation assay. These results are consistent with prior studies that demonstrated different binding epitopes on IGF-1 for receptor and IGFBP interactions (Bayne *et al., supra*, (Vol. 264); Bayne *et al., supra*, (Vol. 265); Cascieri *et al., supra*). The identification of N, N-bis(3-D-gluconamidopropyl)-deoxycholamine as an inhibitor of IGFBP interactions allows the ability to develop small-molecule drugs or peptidomimetics that disrupt the IGF-1/IGFBP complex *in vivo*, thereby releasing receptor-active IGF-1 from the systemic, inactive pool. Such drugs include orally bioavailable therapy for metabolic disease such as diabetes.

Recently, Zeslawski *et al.* (EMBO J., 20: 3638-3644 (2001) published the crystal structure of IGF-1 in complex with the N-terminal domain of IGFBP-5. The structure of that complex is entirely consistent with the model of detergent inhibition of IGFBP binding presented herein, and also disclosed by Vajdos *et al.*, Biochemistry, 40: 11022-11029 (2001). The NMR determination of a complex of IGF-1 bound to a phage-derived IGF-1 antagonist peptide designated IGF-F1-1 (RNCFESVAALRRCMYG (SEQ ID NO:4)), in comparison with other IGF-1 crystal structures, shows that, without limitation to any one theory, a portion of the A-chain (helix III) is mobile in solution, and adopts slightly different conformations when bound to different ligands (detergent, peptide, binding protein).

The complex between peptide IGF-F1-1 and IGF-1 was determined from NMR spectroscopy data collected at 600 and 800 MHz. IGF-1 uniformly labeled with ^{13}C and ^{15}N was prepared using the scheme outlined by Reilly and Fairbrother, J. Biomol. NMR, 4: 459–462 (1994) and purified according to the protocol in Vajdos *et al., supra*. A slight molar excess of unlabeled IGF-F1-1 was mixed with a 1.5 mM solution of $^{13}C/^{15}N$ IGF-1 and ^{1}H, ^{13}C, and ^{15}N NMR resonances assigned from double- and triple-resonance NMR experiments as described by Cavanagh *et al.* in Protein NMR Spectroscopy, Principles and Practice (Academic Press: New York, 1996). Distance restraints within IGF-1 were identified from ^{13}C-edited NOESY HSQC spectra and ^{15}N-edited NOESY HSQC spectra (Cavanagh *et al., supra*).

Intermolecular restraints between IGF-1 and the peptide were obtained from an $\omega 1$-filtered, $\omega 2$-edited ^{13}C HSQC-NOESY spectrum (Lee *et al.*, FEBS Lett., 350: 87-90 (1994)). Intrapeptide distance restraints were obtained from a 2-D ^{13}C-filtered NOESY spectrum. In addition, ϕ dihedral angle restraints were obtained from an HNHA spectrum (Cavanagh *et al., supra*), and $\chi 1$ restraints were derived from HNHB and short-mixing-time TOCSY spectra (Clore *et al.*, J. Biomolec. NMR, 1: 13-22 (1991)). Additional ϕ,ψ restraints were obtained from an analysis of the H^{α}, N, C^{α}, C^{β}, and CO chemical shifts using the program TALOS (Cornilescu *et al.*, J. Biomol. NMR, 13: 289-302 (1999)).

In total, 899 distance restraints (779 intra-IGF-1; 33 intra-peptide; 87 intermolecular), 16 hydrogen bond restraints in helix I, and 138 dihedral angle restraints (71 ϕ; 44 ψ; 23 $\chi 1$) were used to generate an ensemble of structures using a torsion-angle dynamics protocol with the computer program CNX (Accelrys Inc., San Diego). The structure of IGF-1 was well defined for the B-region (residues 2 – 25) and the A-region (residues 41-63) with a mean RMSD from the mean structure for backbone heavy atoms of 0.32 ± 0.06 Å. The C-region (26-40) and the D-region (62-70) were not well defined by the available data. The 20 structures of lowest restraint violation energy had good backbone stereochemistry (80% of residues in the most favored region of ϕ/ψ space with none in disallowed regions) and contained few violations of the experimental restraints (mean maximum distance restraint violation 0.09 ± 0.02 Å). IGF-F1-1 adopts a conformation very similar to that determined for the peptide by itself in solution. The conformation of IGF-1 contains three helices (residues 7-18, 43-49, and 54-60) and is similar to that seen at lower resolution in previous NMR studies of uncomplexed IGF-I (see *e.g.* Cooke *et al., supra*; Sato *et al., supra*; and Laajoki *et al., supra*).

Fig. 10 shows the comparison for the detergent and phage peptide complexes. Specifically, Fig. 10A shows a ribbon diagram of a complex of IGF-1 and N, N-bis(3-D-gluconamidopropyl)-deoxycholamine), and Fig. 10B shows a complex of IGF-1 bound to the phage-derived peptide IGF-F1-1. The B-region (helix I) adopts a very similar conformation in both complexes. The C-loop is only partially ordered in the detergent complex, and ill defined in the peptide complex. Ligand-induced differences are observed for the A-region of IGF-1 (Helix III), at both the backbone (residues 52-60) and side chain (leucine 54 and 57) level. Without limitation to any one theory, maleability in this A-region area is believed to be what allows IGF-1 to bind to so many proteins (six IGFBPs and three receptors).

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the objectives of the present invention.

APPENDIX 1

```
REMARK   3 CRYSTAL STRUCTURE OF IGF-1 SOLVED USING MAD
REMARK   3 REFINEMENT.
REMARK   3  PROGRAM     : X-PLOR(online) 98.1
REMARK   3  AUTHORS     : BRUNGER
REMARK   3
REMARK   3 DATA USED IN REFINEMENT.
REMARK   3  RESOLUTION RANGE HIGH (ANGSTROMS) : 1.80
REMARK   3  RESOLUTION RANGE LOW  (ANGSTROMS) :20.00
REMARK   3  DATA CUTOFF          (SIGMA(F)) : 0.2
REMARK   3  DATA CUTOFF HIGH         (ABS(F)) : 10000000.00
REMARK   3  DATA CUTOFF LOW          (ABS(F)) :  0.001000
REMARK   3  COMPLETENESS (WORKING+TEST)  (%) : 94.8
REMARK   3  NUMBER OF REFLECTIONS          : 6870
REMARK   3
REMARK   3 FIT TO DATA USED IN REFINEMENT.
REMARK   3  CROSS-VALIDATION METHOD        : THROUGHOUT
REMARK   3  FREE R VALUE TEST SET SELECTION  : RANDOM
REMARK   3  R VALUE          (WORKING SET) : 0.237
REMARK   3  FREE R VALUE                   : 0.269
REMARK   3  FREE R VALUE TEST SET SIZE   (%) :  5.6
REMARK   3  FREE R VALUE TEST SET COUNT    :  382
REMARK   3  ESTIMATED ERROR OF FREE R VALUE  : 0.014
REMARK   3
REMARK   3 FIT IN THE HIGHEST RESOLUTION BIN.
REMARK   3  TOTAL NUMBER OF BINS USED        :  6
REMARK   3  BIN RESOLUTION RANGE HIGH      (A) : 1.80
REMARK   3  BIN RESOLUTION RANGE LOW       (A) : 1.91
REMARK   3  BIN COMPLETENESS (WORKING+TEST) (%) : 74.4
REMARK   3  REFLECTIONS IN BIN    (WORKING SET) :  826
REMARK   3  BIN R VALUE          (WORKING SET) : 0.343
REMARK   3  BIN FREE R VALUE                   : 0.439
REMARK   3  BIN FREE R VALUE TEST SET SIZE  (%) :  5.5
REMARK   3  BIN FREE R VALUE TEST SET COUNT    :  48
REMARK   3  ESTIMATED ERROR OF BIN FREE R VALUE : 0.063
REMARK   3
REMARK   3 NUMBER OF NON-HYDROGEN ATOMS USED IN REFINEMENT.
REMARK   3  PROTEIN ATOMS        : 475
REMARK   3  NUCLEIC ACID ATOMS     :  0
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REMARK   3  HETEROGEN ATOMS        :  62
REMARK   3  SOLVENT ATOMS          :  102
REMARK   3
REMARK   3  B VALUES.
REMARK   3  FROM WILSON PLOT        (A**2) : 25.1
REMARK   3  MEAN B VALUE     (OVERALL, A**2) : 33.8
REMARK   3  OVERALL ANISOTROPIC B VALUE.
REMARK   3   B11 (A**2) :  0.00
REMARK   3   B22 (A**2) :  0.00
REMARK   3   B33 (A**2) :  0.00
REMARK   3   B12 (A**2) :  0.00
REMARK   3   B13 (A**2) :  0.00
REMARK   3   B23 (A**2) :  0.00
REMARK   3
REMARK   3  ESTIMATED COORDINATE ERROR.
REMARK   3  ESD FROM LUZZATI PLOT      (A) : 0.23
REMARK   3  ESD FROM SIGMAA            (A) : 0.16
REMARK   3  LOW RESOLUTION CUTOFF      (A) :20.00
REMARK   3
REMARK   3  CROSS-VALIDATED ESTIMATED COORDINATE ERROR.
REMARK   3  ESD FROM C-V LUZZATI PLOT  (A) : 0.28
REMARK   3  ESD FROM C-V SIGMAA        (A) : 0.18
REMARK   3
REMARK   3  RMS DEVIATIONS FROM IDEAL VALUES.
REMARK   3  BOND LENGTHS               (A) : 0.020
REMARK   3  BOND ANGLES          (DEGREES) : 2.0
REMARK   3  DIHEDRAL ANGLES      (DEGREES) : 23.2
REMARK   3  IMPROPER ANGLES      (DEGREES) : 1.09
REMARK   3
REMARK   3  ISOTROPIC THERMAL MODEL : RESTRAINED
REMARK   3
REMARK   3  ISOTROPIC THERMAL FACTOR RESTRAINTS.    RMS    SIGMA
REMARK   3  MAIN-CHAIN BOND          (A**2) :  4.33 ;  4.00
REMARK   3  MAIN-CHAIN ANGLE         (A**2) :  6.46 ;  4.00
REMARK   3  SIDE-CHAIN BOND          (A**2) :  5.41 ;  8.50
REMARK   3  SIDE-CHAIN ANGLE         (A**2) :  8.14 ;  9.00
REMARK   3
REMARK   3  NCS MODEL              : NONE
REMARK   3
REMARK   3  NCS RESTRAINTS.                      RMS    SIGMA/WEIGHT
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REMARK  3  GROUP  1 B-FACTOR          (A**2) : NULL  ; NULL
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REMARK  3 PARAMETER FILE  2  : ../parameter.element
REMARK  3 PARAMETER FILE  3  : ../cyc.par
REMARK  3 PARAMETER FILE  4  : ../../../g2mz/param19.sol
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REMARK  3 TOPOLOGY FILE  2   : ../topology.element
REMARK  3 TOPOLOGY FILE  3   : ../cyc.top
REMARK  3 TOPOLOGY FILE  4   : ../../../g2mz/toph19.sol
REMARK  3
REMARK  3 OTHER REFINEMENT REMARKS: BULK SOLVENT MODEL USED
SEQRES   1 A  100  GLU THR LEU CYS GLY ALA GLU LEU VAL ASP ALA LEU GLN
SEQRES   2 A  100  PHE VAL CYS GLY ASP ARG GLY PHE TYR PHE ASN LYS PRO
SEQRES   3 A  100  THR GLY TYR GLY SER SER THR GLY ILE VAL ASP GLU CYS
SEQRES   4 A  100  CYS PHE ARG SER CYS ASP LEU ARG ARG LEU GLU MET TYR
SEQRES   5 A  100  CYS ALA PRO LEU HOH HOH HOH HOH HOH HOH HOH HOH HOH
SEQRES   6 A  100  HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH
SEQRES   7 A  100  HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH
SEQRES   8 A  100  HOH HOH HOH HOH HOH HOH HOH HOH HOH HOH
SEQRES   1 B    3  CYC BR HOH
SSBOND   1 CYS A    6    CYS A   48
SSBOND   2 CYS A   18    CYS A   61
SSBOND   3 CYS A   47    CYS A   52
CRYST1  31.830   71.074   66.014  90.00  90.00  90.00 C 2 2 21     16
ORIGX1      1.000000  0.000000  0.000000        0.00000
ORIGX2      0.000000  1.000000  0.000000        0.00000
ORIGX3      0.000000  0.000000  1.000000        0.00000
SCALE1      0.031417  0.000000  0.000000        0.00000
SCALE2      0.000000  0.014070  0.000000        0.00000
SCALE3      0.000000  0.000000  0.015148        0.00000
REMARK FILENAME="final.pdb"
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REMARK DATE:Jan-30-01  14:51:37       created by user: mhu
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ATOM     2  CG  GLU A   3      19.515  23.283  21.499  1.00 61.66
ATOM     3  CD  GLU A   3      18.281  24.029  22.025  1.00 70.74
ATOM     4  OE1 GLU A   3      18.254  24.408  23.221  1.00 74.11
ATOM     5  OE2 GLU A   3      17.332  24.237  21.238  1.00 75.50
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ATOM 9 CA GLU A 3 21.994 23.550 20.854 1.00 40.66
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ATOM 11 CA THR A 4 24.213 20.754 22.059 1.00 24.88
ATOM 12 CB THR A 4 25.301 20.217 21.051 1.00 23.86
ATOM 13 OG1 THR A 4 26.426 21.106 21.070 1.00 30.08
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ATOM 24 O LEU A 5 27.742 18.527 24.980 1.00 20.58
ATOM 25 N CYS A 6 28.352 20.591 25.572 1.00 19.74
ATOM 26 CA CYS A 6 29.809 20.320 25.520 1.00 21.84
ATOM 27 C CYS A 6 30.485 20.754 26.790 1.00 16.04
ATOM 28 O CYS A 6 29.990 21.602 27.535 1.00 18.05
ATOM 29 CB CYS A 6 30.448 21.077 24.361 1.00 22.37
ATOM 30 SG CYS A 6 29.753 20.589 22.733 1.00 32.91
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ATOM 32 CA GLY A 7 32.397 20.613 28.217 1.00 17.15
ATOM 33 C GLY A 7 31.698 20.668 29.536 1.00 18.61
ATOM 34 O GLY A 7 31.069 19.683 29.965 1.00 15.50
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ATOM 42 CB GLU A 9 27.118 22.975 29.140 1.00 23.00
ATOM 43 CG GLU A 9 27.116 24.481 29.346 1.00 33.94
ATOM 44 CD GLU A 9 26.424 25.279 28.204 1.00 53.09
ATOM 45 OE1 GLU A 9 25.790 24.700 27.260 1.00 50.65

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ATOM   67 CG  ASP A 12    26.960 22.337 34.970 1.00 27.52
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ATOM 104 O PHE A 16 20.248 15.013 37.971 1.00 20.37
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ATOM 106 CA VAL A 17 20.338 13.761 35.490 1.00 17.94
ATOM 107 CB VAL A 17 20.392 13.598 33.932 1.00 21.28
ATOM 108 CG1 VAL A 17 19.831 12.219 33.456 1.00 25.51
ATOM 109 CG2 VAL A 17 19.619 14.737 33.295 1.00 22.15
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ATOM 125 CG ASP A 20 23.633 10.169 45.437 1.00 73.16

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ATOM 129 O ASP A 20 25.954 8.521 42.472 1.00 38.02
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ATOM 138 NH2 ARG A 21 20.103 6.474 36.138 1.00 34.61
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ATOM 149 CD1 PHE A 23 28.271 11.908 35.762 1.00 24.30
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ATOM 153 CZ PHE A 23 30.259 13.307 35.458 1.00 22.56
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ATOM  167  O  TYR A 24    29.264   8.859  30.221  1.00 31.49
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ATOM  169  CA  PHE A 25   31.546   9.091  28.625  1.00 18.28
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ATOM  174  CE1 PHE A 25   33.264  12.190  31.432  1.00 25.64
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ATOM  176  CZ  PHE A 25   32.794  13.394  30.888  1.00 27.30
ATOM  177  C  PHE A 25    31.385   8.046  27.534  1.00 20.94
ATOM  178  O  PHE A 25    30.992   8.341  26.411  1.00 21.90
ATOM  179  N  ASN A 26    31.708   6.793  27.868  1.00 25.83
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ATOM  182  CG  ASN A 26   33.737   6.469  25.737  1.00 29.50
ATOM  183  OD1 ASN A 26   34.438   7.267  26.395  1.00 38.35
ATOM  184  ND2 ASN A 26   33.508   6.626  24.462  1.00 32.35
ATOM  185  C  ASN A 26    30.924   4.534  27.633  1.00 23.53
ATOM  186  O  ASN A 26    31.274   4.214  28.753  1.00 23.60
ATOM  187  N  LYS A 27    29.958   3.879  27.025  1.00 26.93
ATOM  188  CA  LYS A 27   29.325   2.795  27.791  1.00 33.05
ATOM  189  CB  LYS A 27   28.018   2.384  27.113  1.00 34.53
ATOM  190  CG  LYS A 27   27.014   3.521  27.084  1.00 37.05
ATOM  191  CD  LYS A 27   26.126   3.416  25.878  1.00 39.60
ATOM  192  CE  LYS A 27   24.768   4.078  26.139  1.00 42.35
ATOM  193  NZ  LYS A 27   24.244   4.674  24.865  1.00 39.82
ATOM  194  C  LYS A 27    30.242   1.603  27.870  1.00 31.68
ATOM  195  O  LYS A 27    30.732   1.173  26.848  1.00 31.77
ATOM  196  N  PRO A 28    30.494   1.065  29.073  1.00 35.47
ATOM  197  CD  PRO A 28   30.001   1.491  30.392  1.00 34.96
ATOM  198  CA  PRO A 28   31.388  -0.110  29.148  1.00 36.66
ATOM  199  CB  PRO A 28   31.468  -0.435  30.637  1.00 40.26
ATOM  200  CG  PRO A 28   30.367   0.359  31.287  1.00 40.80
ATOM  201  C  PRO A 28    30.784  -1.252  28.322  1.00 36.18
ATOM  202  O  PRO A 28    29.558  -1.344  28.152  1.00 36.79
ATOM  203  N  THR A 29    31.614  -2.107  27.763  1.00 34.63
ATOM  204  CA  THR A 29   31.026  -3.155  26.945  1.00 41.92
ATOM  205  CB  THR A 29   31.824  -3.391  25.661  1.00 51.94
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ATOM 206 OG1 THR A 29 32.938 -4.249 25.947 1.00 52.79
ATOM 207 CG2 THR A 29 32.313 -2.071 25.086 1.00 55.75
ATOM 208 C THR A 29 30.897 -4.498 27.635 1.00 35.36
ATOM 209 O THR A 29 30.068 -5.322 27.238 1.00 34.58
ATOM 210 N GLY A 30 31.721 -4.720 28.643 1.00 33.36
ATOM 211 CA GLY A 30 31.659 -5.997 29.323 1.00 33.97
ATOM 212 C GLY A 30 32.109 -7.243 28.536 1.00 31.86
ATOM 213 O GLY A 30 32.227 -7.275 27.305 1.00 34.94
ATOM 214 N TYR A 31 32.265 -8.325 29.275 1.00 23.05
ATOM 215 CA TYR A 31 32.723 -9.576 28.690 1.00 25.72
ATOM 216 CB TYR A 31 33.144 -10.505 29.813 1.00 21.15
ATOM 217 CG TYR A 31 34.274 -9.992 30.633 1.00 24.03
ATOM 218 CD1 TYR A 31 34.066 -9.497 31.892 1.00 17.01
ATOM 219 CE1 TYR A 31 35.106 -8.997 32.644 1.00 26.09
ATOM 220 CD2 TYR A 31 35.579 -9.983 30.121 1.00 29.13
ATOM 221 CE2 TYR A 31 36.616 -9.502 30.870 1.00 25.81
ATOM 222 CZ TYR A 31 36.383 -9.009 32.115 1.00 25.77
ATOM 223 OH TYR A 31 37.419 -8.560 32.875 1.00 34.26
ATOM 224 C TYR A 31 31.678 -10.274 27.840 1.00 29.45
ATOM 225 O TYR A 31 30.468 -10.172 28.112 1.00 30.08
ATOM 226 N GLY A 32 32.141 -10.990 26.808 1.00 29.62
ATOM 227 CA GLY A 32 31.228 -11.746 25.972 1.00 33.28
ATOM 228 C GLY A 32 30.235 -10.912 25.198 1.00 36.13
ATOM 229 O GLY A 32 29.161 -11.380 24.832 1.00 32.23
ATOM 230 N SER A 33 30.606 -9.668 24.937 1.00 41.08
ATOM 231 CA SER A 33 29.722 -8.787 24.217 1.00 47.10
ATOM 232 CB SER A 33 30.130 -7.331 24.386 1.00 46.73
ATOM 233 OG SER A 33 29.397 -6.557 23.463 1.00 52.64
ATOM 234 C SER A 33 29.816 -9.142 22.764 1.00 57.93
ATOM 235 O SER A 33 30.807 -9.735 22.317 1.00 57.01
ATOM 236 N SER A 34 28.772 -8.755 22.039 1.00 65.04
ATOM 237 CA SER A 34 28.657 -8.989 20.613 1.00 70.41
ATOM 238 CB SER A 34 28.414 -7.659 19.899 1.00 72.65
ATOM 239 OG SER A 34 27.049 -7.299 19.995 1.00 72.85
ATOM 240 C SER A 34 29.885 -9.671 20.028 1.00 71.58
ATOM 241 O SER A 34 30.642 -9.053 19.289 1.00 69.77
ATOM 242 CB THR A 41 30.810 6.812 19.043 1.00 59.11
ATOM 243 OG1 THR A 41 29.666 5.952 18.975 1.00 64.04
ATOM 244 CG2 THR A 41 31.511 6.892 17.700 1.00 59.18
ATOM 245 C THR A 41 31.044 6.416 21.449 1.00 51.54

ATOM 246 O THR A 41 30.689 5.415 22.079 1.00 55.44
ATOM 247 N THR A 41 32.206 4.887 19.817 1.00 58.60
ATOM 248 CA THR A 41 31.763 6.289 20.105 1.00 57.60
ATOM 249 N GLY A 42 30.804 7.654 21.870 1.00 35.08
ATOM 250 CA GLY A 42 30.159 7.853 23.151 1.00 21.75
ATOM 251 C GLY A 42 29.409 9.184 23.225 1.00 18.36
ATOM 252 O GLY A 42 29.011 9.724 22.200 1.00 21.32
ATOM 253 N ILE A 43 29.298 9.708 24.431 1.00 21.07
ATOM 254 CA ILE A 43 28.475 10.899 24.619 1.00 22.17
ATOM 255 CB ILE A 43 28.247 11.113 26.148 1.00 18.01
ATOM 256 CG2 ILE A 43 29.474 11.764 26.819 1.00 20.05
ATOM 257 CG1 ILE A 43 27.124 12.102 26.413 1.00 20.02
ATOM 258 CD1 ILE A 43 26.793 12.170 27.882 1.00 22.13
ATOM 259 C ILE A 43 28.947 12.145 23.921 1.00 25.61
ATOM 260 O ILE A 43 28.118 12.955 23.466 1.00 18.04
ATOM 261 N VAL A 44 30.260 12.336 23.748 1.00 19.35
ATOM 262 CA VAL A 44 30.629 13.560 23.090 1.00 19.83
ATOM 263 CB VAL A 44 32.187 13.809 23.157 1.00 22.51
ATOM 264 CG1 VAL A 44 32.587 14.916 22.238 1.00 29.90
ATOM 265 CG2 VAL A 44 32.568 14.056 24.605 1.00 22.31
ATOM 266 C VAL A 44 30.137 13.492 21.655 1.00 18.37
ATOM 267 O VAL A 44 29.664 14.477 21.127 1.00 20.08
ATOM 268 N ASP A 45 30.257 12.308 21.027 1.00 19.78
ATOM 269 CA ASP A 45 29.821 12.142 19.657 1.00 22.36
ATOM 270 CB ASP A 45 30.132 10.738 19.207 1.00 29.90
ATOM 271 CG ASP A 45 31.588 10.395 19.435 1.00 38.30
ATOM 272 OD1 ASP A 45 32.374 10.700 18.516 1.00 34.84
ATOM 273 OD2 ASP A 45 31.929 9.881 20.546 1.00 39.04
ATOM 274 C ASP A 45 28.304 12.345 19.531 1.00 23.03
ATOM 275 O ASP A 45 27.830 13.023 18.613 1.00 22.16
ATOM 276 N GLU A 46 27.610 11.772 20.489 1.00 21.13
ATOM 277 CA GLU A 46 26.139 11.784 20.505 1.00 24.96
ATOM 278 CB GLU A 46 25.676 10.660 21.418 1.00 32.84
ATOM 279 CG GLU A 46 24.220 10.314 21.307 1.00 38.09
ATOM 280 CD GLU A 46 23.877 8.973 21.913 1.00 41.51
ATOM 281 OE1 GLU A 46 24.713 8.378 22.621 1.00 41.76
ATOM 282 OE2 GLU A 46 22.739 8.505 21.689 1.00 45.60
ATOM 283 C GLU A 46 25.472 13.084 20.952 1.00 24.92
ATOM 284 O GLU A 46 24.450 13.450 20.376 1.00 23.35
ATOM 285 N CYS A 47 26.045 13.747 21.970 1.00 20.20

ATOM 286 CA CYS A 47 25.477 14.961 22.590 1.00 19.56
ATOM 287 C CYS A 47 26.174 16.282 22.474 1.00 28.23
ATOM 288 O CYS A 47 25.563 17.328 22.769 1.00 21.99
ATOM 289 CB CYS A 47 25.240 14.687 24.091 1.00 21.60
ATOM 290 SG CYS A 47 24.245 13.186 24.373 1.00 26.79
ATOM 291 N CYS A 48 27.452 16.263 22.074 1.00 18.45
ATOM 292 CA CYS A 48 28.181 17.537 21.940 1.00 17.21
ATOM 293 C CYS A 48 28.382 17.818 20.473 1.00 19.88
ATOM 294 O CYS A 48 28.082 18.890 20.040 1.00 23.54
ATOM 295 CB CYS A 48 29.521 17.456 22.686 1.00 22.56
ATOM 296 SG CYS A 48 30.678 18.814 22.279 1.00 25.89
ATOM 297 N PHE A 49 28.854 16.830 19.706 1.00 19.44
ATOM 298 CA PHE A 49 29.044 17.010 18.279 1.00 26.47
ATOM 299 CB PHE A 49 29.939 15.912 17.710 1.00 27.72
ATOM 300 CG PHE A 49 31.343 15.940 18.268 1.00 32.07
ATOM 301 CD1 PHE A 49 32.098 14.764 18.363 1.00 31.76
ATOM 302 CD2 PHE A 49 31.902 17.133 18.703 1.00 29.85
ATOM 303 CE1 PHE A 49 33.396 14.798 18.889 1.00 32.64
ATOM 304 CE2 PHE A 49 33.203 17.153 19.230 1.00 29.98
ATOM 305 CZ PHE A 49 33.938 15.987 19.320 1.00 24.87
ATOM 306 C PHE A 49 27.706 17.000 17.573 1.00 27.42
ATOM 307 O PHE A 49 27.561 17.598 16.517 1.00 31.83
ATOM 308 N ARG A 50 26.744 16.298 18.167 1.00 29.59
ATOM 309 CA ARG A 50 25.381 16.219 17.640 1.00 26.05
ATOM 310 CB ARG A 50 25.106 14.832 17.157 1.00 23.82
ATOM 311 CG ARG A 50 25.916 14.488 15.899 1.00 26.06
ATOM 312 CD ARG A 50 25.953 13.031 15.742 1.00 29.51
ATOM 313 NE ARG A 50 26.583 12.750 14.479 1.00 39.53
ATOM 314 CZ ARG A 50 27.849 12.386 14.370 1.00 41.06
ATOM 315 NH1 ARG A 50 28.603 12.260 15.466 1.00 40.37
ATOM 316 NH2 ARG A 50 28.354 12.198 13.163 1.00 40.98
ATOM 317 C ARG A 50 24.434 16.539 18.794 1.00 25.89
ATOM 318 O ARG A 50 24.864 16.563 19.966 1.00 19.42
ATOM 319 N SER A 51 23.161 16.811 18.501 1.00 25.83
ATOM 320 CA SER A 51 22.229 17.080 19.633 1.00 28.49
ATOM 321 CB SER A 51 21.122 18.082 19.248 1.00 29.44
ATOM 322 OG SER A 51 20.679 17.787 17.954 1.00 42.62
ATOM 323 C SER A 51 21.569 15.761 20.025 1.00 22.34
ATOM 324 O SER A 51 21.229 14.963 19.164 1.00 28.05
ATOM 325 N CYS A 52 21.385 15.517 21.325 1.00 21.96

ATOM 326 CA CYS A 52 20.771 14.278 21.762 1.00 24.73
ATOM 327 C CYS A 52 19.637 14.683 22.708 1.00 25.51
ATOM 328 O CYS A 52 19.616 15.780 23.240 1.00 23.80
ATOM 329 CB CYS A 52 21.791 13.373 22.494 1.00 24.55
ATOM 330 SG CYS A 52 22.331 13.922 24.151 1.00 28.05
ATOM 331 N ASP A 53 18.671 13.815 22.896 1.00 27.35
ATOM 332 CA ASP A 53 17.622 14.228 23.789 1.00 28.62
ATOM 333 CB ASP A 53 16.248 13.872 23.201 1.00 34.84
ATOM 334 CG ASP A 53 16.078 12.408 22.968 1.00 39.80
ATOM 335 OD1 ASP A 53 16.598 11.616 23.782 1.00 44.65
ATOM 336 OD2 ASP A 53 15.413 12.052 21.965 1.00 46.35
ATOM 337 C ASP A 53 17.848 13.616 25.147 1.00 23.60
ATOM 338 O ASP A 53 18.735 12.762 25.324 1.00 21.40
ATOM 339 N LEU A 54 17.012 14.005 26.099 1.00 20.38
ATOM 340 CA LEU A 54 17.163 13.558 27.477 1.00 14.25
ATOM 341 CB LEU A 54 16.015 14.169 28.329 1.00 21.53
ATOM 342 CG LEU A 54 16.024 13.807 29.805 1.00 22.22
ATOM 343 CD1 LEU A 54 17.283 14.383 30.476 1.00 21.85
ATOM 344 CD2 LEU A 54 14.733 14.336 30.497 1.00 20.36
ATOM 345 C LEU A 54 17.276 12.046 27.683 1.00 23.79
ATOM 346 O LEU A 54 18.075 11.598 28.496 1.00 23.74
ATOM 347 N ARG A 55 16.449 11.253 26.978 1.00 23.24
ATOM 348 CA ARG A 55 16.489 9.795 27.104 1.00 26.67
ATOM 349 CB ARG A 55 15.469 9.140 26.140 1.00 33.74
ATOM 350 CG ARG A 55 14.013 9.234 26.618 1.00 47.08
ATOM 351 CD ARG A 55 13.838 8.500 27.974 1.00 59.43
ATOM 352 NE ARG A 55 13.580 9.398 29.104 1.00 68.64
ATOM 353 CZ ARG A 55 14.359 9.527 30.182 1.00 73.73
ATOM 354 NH1 ARG A 55 15.485 8.813 30.302 1.00 74.93
ATOM 355 NH2 ARG A 55 14.028 10.401 31.134 1.00 72.23
ATOM 356 C ARG A 55 17.876 9.229 26.774 1.00 25.72
ATOM 357 O ARG A 55 18.365 8.348 27.470 1.00 28.77
ATOM 358 N ARG A 56 18.482 9.765 25.726 1.00 21.19
ATOM 359 CA ARG A 56 19.785 9.285 25.276 1.00 27.70
ATOM 360 CB ARG A 56 20.049 9.798 23.892 1.00 35.27
ATOM 361 CG ARG A 56 19.140 9.134 22.892 1.00 42.72
ATOM 362 CD ARG A 56 19.352 7.624 22.897 1.00 52.32
ATOM 363 NE ARG A 56 20.565 7.280 22.150 1.00 59.61
ATOM 364 CZ ARG A 56 21.142 6.075 22.108 1.00 61.66
ATOM 365 NH1 ARG A 56 20.646 5.046 22.789 1.00 64.42

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ATOM   366 NH2 ARG A 56     22.244   5.906  21.389  1.00 62.77
ATOM   367 C   ARG A 56     20.850   9.724  26.246  1.00 25.14
ATOM   368 O   ARG A 56     21.755   8.952  26.618  1.00 25.99
ATOM   369 N   LEU A 57     20.743  10.967  26.689  1.00 21.87
ATOM   370 CA  LEU A 57     21.684  11.448  27.701  1.00 22.79
ATOM   371 CB  LEU A 57     21.367  12.923  28.059  1.00 22.01
ATOM   372 CG  LEU A 57     22.188  13.715  29.089  1.00 22.86
ATOM   373 CD1 LEU A 57     21.537  15.099  29.190  1.00 27.83
ATOM   374 CD2 LEU A 57     22.115  13.120  30.453  1.00 32.64
ATOM   375 C   LEU A 57     21.665  10.578  28.970  1.00 20.53
ATOM   376 O   LEU A 57     22.720  10.208  29.525  1.00 19.79
ATOM   377 N   GLU A 58     20.486  10.199  29.479  1.00 18.07
ATOM   378 CA  GLU A 58     20.425   9.390  30.682  1.00 17.97
ATOM   379 CB  GLU A 58     18.951   9.351  31.198  1.00 24.34
ATOM   380 CG  GLU A 58     18.883   8.941  32.660  1.00 36.31
ATOM   381 CD  GLU A 58     17.473   9.016  33.227  1.00 41.86
ATOM   382 OE1 GLU A 58     17.316   8.902  34.474  1.00 39.29
ATOM   383 OE2 GLU A 58     16.549   9.198  32.406  1.00 36.76
ATOM   384 C   GLU A 58     21.005   7.942  30.537  1.00 14.19
ATOM   385 O   GLU A 58     21.331   7.279  31.508  1.00 26.60
ATOM   386 N   MET A 59     21.172   7.487  29.307  1.00 15.83
ATOM   387 CA  MET A 59     21.767   6.166  29.115  1.00 21.59
ATOM   388 CB  MET A 59     21.626   5.746  27.672  1.00 23.71
ATOM   389 CG  MET A 59     20.195   5.145  27.372  1.00 27.46
ATOM   390 SD  MET A 59     19.916   5.067  25.648  1.00 38.20
ATOM   391 CE  MET A 59     18.000   5.126  25.597  1.00 38.66
ATOM   392 C   MET A 59     23.261   6.169  29.521  1.00 23.34
ATOM   393 O   MET A 59     23.859   5.124  29.663  1.00 25.38
ATOM   394 N   TYR A 60     23.847   7.353  29.726  1.00 19.16
ATOM   395 CA  TYR A 60     25.266   7.386  30.144  1.00 18.17
ATOM   396 CB  TYR A 60     25.982   8.549  29.452  1.00 18.20
ATOM   397 CG  TYR A 60     26.144   8.364  27.992  1.00 20.05
ATOM   398 CD1 TYR A 60     25.193   8.855  27.086  1.00 20.40
ATOM   399 CE1 TYR A 60     25.339   8.675  25.713  1.00 22.99
ATOM   400 CD2 TYR A 60     27.245   7.686  27.482  1.00 23.58
ATOM   401 CE2 TYR A 60     27.398   7.501  26.131  1.00 24.88
ATOM   402 CZ  TYR A 60     26.475   7.980  25.258  1.00 25.27
ATOM   403 OH  TYR A 60     26.676   7.780  23.940  1.00 24.52
ATOM   404 C   TYR A 60     25.406   7.424  31.634  1.00 22.93
ATOM   405 O   TYR A 60     26.519   7.507  32.199  1.00 22.51
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ATOM 406 N CYS A 61 24.290 7.385 32.352 1.00 18.42
ATOM 407 CA CYS A 61 24.402 7.303 33.809 1.00 15.63
ATOM 408 C CYS A 61 24.808 5.872 34.207 1.00 19.70
ATOM 409 O CYS A 61 24.394 4.908 33.563 1.00 25.04
ATOM 410 CB CYS A 61 23.065 7.562 34.511 1.00 22.45
ATOM 411 SG CYS A 61 22.415 9.181 34.212 1.00 24.27
ATOM 412 N ALA A 62 25.543 5.738 35.298 1.00 24.74
ATOM 413 CA ALA A 62 26.004 4.424 35.756 1.00 31.14
ATOM 414 CB ALA A 62 27.273 4.588 36.641 1.00 24.85
ATOM 415 C ALA A 62 24.902 3.747 36.563 1.00 36.05
ATOM 416 O ALA A 62 23.920 4.394 36.962 1.00 33.02
ATOM 417 N PRO A 63 25.014 2.427 36.780 1.00 44.06
ATOM 418 CD PRO A 63 26.021 1.447 36.310 1.00 47.05
ATOM 419 CA PRO A 63 23.942 1.803 37.576 1.00 46.88
ATOM 420 CB PRO A 63 24.182 0.296 37.393 1.00 47.68
ATOM 421 CG PRO A 63 25.651 0.166 37.068 1.00 49.88
ATOM 422 C PRO A 63 24.111 2.253 39.027 1.00 48.13
ATOM 423 O PRO A 63 23.135 2.412 39.773 1.00 54.36
ATOM 424 N LEU A 64 25.379 2.467 39.379 1.00 51.56
ATOM 425 CA LEU A 64 25.835 2.896 40.693 1.00 61.37
ATOM 426 CB LEU A 64 26.997 1.994 41.170 1.00 63.79
ATOM 427 CG LEU A 64 26.761 0.984 42.312 1.00 60.23
ATOM 428 CD1 LEU A 64 26.872 -0.435 41.767 1.00 61.06
ATOM 429 CD2 LEU A 64 27.781 1.210 43.447 1.00 58.43
ATOM 430 C LEU A 64 26.327 4.343 40.607 1.00 67.33
ATOM 431 O LEU A 64 27.441 4.612 40.132 1.00 71.54
ATOM 432 C1 CYC B 1 19.808 21.082 25.297 1.00 29.47
ATOM 433 C6 CYC B 1 19.974 19.730 24.564 1.00 31.45
ATOM 434 O21 CYC B 1 21.316 19.561 24.000 1.00 30.05
ATOM 435 C5 CYC B 1 19.707 18.570 25.545 1.00 24.70
ATOM 436 C4 CYC B 1 18.349 18.647 26.185 1.00 28.97
ATOM 437 C11 CYC B 1 18.069 17.503 27.203 1.00 25.55
ATOM 438 C10 CYC B 1 18.915 17.625 28.449 1.00 26.59
ATOM 439 C9 CYC B 1 18.774 19.062 29.167 1.00 22.28
ATOM 440 C15 CYC B 1 19.668 19.202 30.407 1.00 18.50
ATOM 441 C18 CYC B 1 19.488 18.173 31.529 1.00 17.96
ATOM 442 C17 CYC B 1 20.033 18.861 32.738 1.00 20.63
ATOM 443 C16 CYC B 1 20.478 20.370 32.210 1.00 19.19
ATOM 444 C43 CYC B 1 20.408 21.271 33.357 1.00 17.91
ATOM 445 C55 CYC B 1 21.476 20.723 34.438 1.00 26.47

ATOM 446 C56 CYC B 1 21.498 21.442 35.738 1.00 31.90
ATOM 447 C57 CYC B 1 22.417 22.653 36.015 1.00 39.26
ATOM 448 N59 CYC B 1 22.473 23.332 37.133 1.00 46.97
ATOM 449 C74 CYC B 1 23.443 24.507 37.216 1.00 56.76
ATOM 450 C75 CYC B 1 22.897 25.835 36.400 1.00 62.01
ATOM 451 C76 CYC B 1 23.920 27.038 36.528 1.00 66.74
ATOM 452 N77 CYC B 1 23.715 27.349 37.968 1.00 68.00
ATOM 453 C78 CYC B 1 24.623 27.135 39.017 1.00 72.42
ATOM 454 C80 CYC B 1 24.134 27.596 40.383 1.00 76.46
ATOM 455 O86 CYC B 1 22.731 28.037 40.272 1.00 73.03
ATOM 456 C81 CYC B 1 25.194 28.755 40.862 1.00 85.35
ATOM 457 O87 CYC B 1 25.451 29.858 39.876 1.00 86.03
ATOM 458 C82 CYC B 1 24.767 29.384 42.284 1.00 92.20
ATOM 459 O88 CYC B 1 23.400 29.939 42.115 1.00 92.22
ATOM 460 C83 CYC B 1 25.777 30.529 42.728 1.00 96.59
ATOM 461 O89 CYC B 1 27.124 29.924 42.873 1.00 99.38
ATOM 462 C84 CYC B 1 25.395 31.205 44.130 1.00 97.03
ATOM 463 O85 CYC B 1 26.318 32.274 44.541 1.00 95.57
ATOM 464 O79 CYC B 1 25.765 26.665 38.880 1.00 74.76
ATOM 465 C68 CYC B 1 21.737 23.120 38.324 1.00 39.41
ATOM 466 C69 CYC B 1 20.223 23.496 38.137 1.00 38.89
ATOM 467 C70 CYC B 1 19.523 23.355 39.421 1.00 38.22
ATOM 468 N71 CYC B 1 20.564 22.987 40.353 1.00 50.02
ATOM 469 C72 CYC B 1 20.399 22.763 41.607 1.00 55.62
ATOM 470 C90 CYC B 1 21.157 21.536 42.121 1.00 57.83
ATOM 471 O96 CYC B 1 20.407 20.319 41.993 1.00 56.55
ATOM 472 C91 CYC B 1 21.664 21.940 43.578 1.00 63.18
ATOM 473 O97 CYC B 1 21.600 20.757 44.471 1.00 63.88
ATOM 474 C92 CYC B 1 23.186 22.499 43.622 1.00 66.58
ATOM 475 O98 CYC B 1 23.971 21.462 44.307 1.00 68.12
ATOM 476 C93 CYC B 1 23.135 23.867 44.446 1.00 67.49
ATOM 477 O99 CYC B 1 22.357 24.774 43.597 1.00 66.74
ATOM 478 C94 CYC B 1 24.493 24.656 44.683 1.00 74.63
ATOM 479 O95 CYC B 1 24.178 25.865 45.490 1.00 80.12
ATOM 480 O73 CYC B 1 19.735 23.526 42.349 1.00 63.35
ATOM 481 O58 CYC B 1 23.106 22.977 35.125 1.00 40.42
ATOM 482 C54 CYC B 1 20.742 22.893 32.977 1.00 18.54
ATOM 483 C14 CYC B 1 19.521 20.648 31.087 1.00 23.61
ATOM 484 C19 CYC B 1 17.911 20.963 31.644 1.00 16.19
ATOM 485 C13 CYC B 1 19.810 21.781 30.035 1.00 19.97

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ATOM  486 O20 CYC B   1     21.223 21.655 29.535 1.00 19.41
ATOM  487 C12 CYC B   1     18.975 21.668 28.869 1.00 17.80
ATOM  488 C8  CYC B   1     19.095 20.197 28.184 1.00 20.86
ATOM  489 C3  CYC B   1     18.129 20.072 26.867 1.00 26.70
ATOM  490 C7  CYC B   1     16.492 20.310 27.375 1.00 27.90
ATOM  491 C2  CYC B   1     18.448 21.212 25.824 1.00 28.19
ATOM  492 BR  BR  C   1     34.062  6.612 30.395 0.50 33.08
ATOM  493 O   HOH W   1     19.167 24.790 26.224 1.00 26.47
ATOM  494 O   HOH W   2     23.897 21.958 31.132 1.00 22.60
ATOM  495 O   HOH W   3     21.553 23.642 27.485 1.00 29.42
ATOM  496 O   HOH W   4     24.680 19.970 37.794 1.00 27.43
ATOM  497 O   HOH W   5     30.918 19.401 40.676 1.00 23.25
ATOM  498 O   HOH W   6     16.708 17.200 40.896 1.00 28.59
ATOM  499 O   HOH W   7     16.516 25.080 41.353 1.00 25.88
ATOM  500 O   HOH W   8     18.741 11.412 20.841 1.00 29.70
ATOM  501 O   HOH W   9     27.307 23.459 25.693 1.00 22.59
ATOM  502 O   HOH W  10     29.313 -4.298 41.568 1.00 37.18
ATOM  503 O   HOH W  11     24.485 23.989 32.854 1.00 35.58
ATOM  504 O   HOH W  12     17.430 11.457 36.970 1.00 26.36
ATOM  505 O   HOH W  13     22.518 16.744 15.421 1.00 37.65
ATOM  506 O   HOH W  14     22.764 17.648 22.807 1.00 29.54
ATOM  507 O   HOH W  15     22.916 12.486 18.554 1.00 30.85
ATOM  508 O   HOH W  16     21.127 13.160 16.233 1.00 67.05
ATOM  509 O   HOH W  18     33.941  3.332 34.867 1.00 47.13
ATOM  510 O   HOH W  19     28.659 24.132 37.361 1.00 34.15
ATOM  511 O   HOH W  20     26.049 12.100 38.687 1.00 43.38
ATOM  512 O   HOH W  22     22.998  5.484 43.119 1.00 56.07
ATOM  513 O   HOH W  23     21.091 10.590 19.804 1.00 37.65
ATOM  514 O   HOH W  24     27.553 20.681 37.920 1.00 40.51
ATOM  515 O   HOH W  25     21.539  4.646 32.538 1.00 35.17
ATOM  516 O   HOH W  35     36.200  4.986 34.099 1.00 55.94
ATOM  517 O   HOH W  36     25.301 25.430 41.805 1.00 45.43
ATOM  518 O   HOH W  37     28.063 14.145 40.069 1.00 39.11
ATOM  519 O   HOH W  38     20.561 25.768 50.428 1.00 38.92
ATOM  520 O   HOH W  39     22.841 23.744 25.080 1.00 40.88
ATOM  521 O   HOH W  40     16.781 11.726 39.998 1.00 65.62
ATOM  522 O   HOH W  41     29.705 23.094 40.234 1.00 40.48
ATOM  523 O   HOH W  42     18.375 14.116 41.324 1.00 58.97
ATOM  524 O   HOH W  43     15.538  9.778 20.639 1.00 61.25
ATOM  525 O   HOH W  44     34.144  2.745 20.168 1.00 48.45
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ATOM	526	O	HOH	W	45	20.621	8.952	42.429	1.00	33.04
ATOM	527	O	HOH	W	46	17.087	6.329	28.850	1.00	32.70
ATOM	528	O	HOH	W	47	25.800	23.668	39.622	1.00	46.80
ATOM	529	O	HOH	W	48	16.978	27.199	25.066	1.00	44.77
ATOM	530	O	HOH	W	49	22.764	7.118	24.736	1.00	38.06
ATOM	531	O	HOH	W	50	24.770	22.332	46.924	1.00	48.70
ATOM	532	O	HOH	W	51	21.688	25.678	41.327	1.00	52.53
ATOM	533	O	HOH	W	52	19.396	17.922	42.396	1.00	53.49
ATOM	534	O	HOH	W	53	21.375	18.431	46.997	1.00	57.91
ATOM	535	O	HOH	W	54	19.736	20.961	17.193	1.00	46.33
ATOM	536	O	HOH	W	55	30.374	4.915	45.161	1.00	52.15
ATOM	537	O	HOH	W	56	18.588	26.408	48.436	1.00	44.58
ATOM	538	O	HOH	W	57	23.722	24.504	28.990	1.00	38.53